

PRVPATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

REC'D 09.AUG 2004

WIPO

PCT

**Intyg
Certificate**

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

Ansökan ingavs ursprungligen på engelska.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

The application was originally filed in English.

(71) Sökande AstraZeneca AB, Södertälje SE
Applicant (s)

(21) Patentansökningsnummer 0302122-7
Patent application number

(86) Ingivningsdatum 2003-07-22
Date of filing

Stockholm, 2004-06-30

För Patent- och registreringsverket
For the Patent- and Registration Office

Hjärdis Segerlund

Avgift

Fee 170:-

**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

**PATENT- OCH
REGISTRERINGSVERKET
SWEDEN**

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

BEST AVAILABLE COPY

-1-

FIELD OF THE INVENTION

The present invention describes a coronary artery disease susceptibility gene and its use in the diagnosis and therapy of CAD.

BACKGROUND

Coronary artery disease (CAD) is the most common type of heart disease. It is a consequence of atherosclerosis, which is a progressive inflammatory tissue response resulting from deposition of modified lipoproteins in the arterial intima of the vascular wall. This process can lead to development of complex atherosclerotic lesions or plaques at pre-existing focal arterial intima cushions rich in extracellular matrix (Glass *et al*, Cell 104, 503-516, 2001). Overtime these lesions narrow the coronary arteries, restricting blood flow to the heart and can cause angina. Complete blockage can lead to myocardial infarction.

The known risk factors for CAD include type II diabetes, insulin resistance, obesity (Rao *et al*, Am. Heart J. 142, 1102-1107, 2001), hyperlipidemia, high blood pressure, cigarette smoking and physical inactivity. CAD is also known to have a significant genetic component.

There are no drugs currently available to prevent CAD. Efforts have been focussed at measures to slow down CAD, and different drugs and surgical techniques are available to repair clogged coronary arteries. However, there is a need for a better understanding of the pathophysiology of CAD and the development of specific and effective drugs.

To develop more effective drugs, it is critical that the susceptibility genes for CAD are identified. As mentioned above, the pathogenesis of CAD has been shown by twin and other studies to include a significant genetic component, and the effects of genes on susceptibility to CAD are likely to be particularly strong at younger ages (Marenberg *et al*, New England J Med 330, 1041-1046, 1994). However, CAD is unlikely to be inherited as a simple Mendelian trait, but is instead a complex multifactorial disorder in which the phenotype is heavily influenced by environmental as well as genetic factors. Although a number of loci have been identified for risk factors related to CAD, only a limited number of genome

-2-

scanning studies have been published showing linkage to CAD. For example, CAD has been linked to chromosome 2 and X (Pajukanta et al, Am. J. Hum. Genet. 67, 1481-1493, 2000), chromosome 16 (Francke *et al.*, Human Molec. Genet. 10, 2751-2765, 2001) and chromosome 14 (Broeckel *et al.*, Nature Genetics 30, 210, 2002).

SUMMARY OF THE INVENTION

The present invention describes the identification of a CAD susceptibility gene, a basic helix-loop-helix transcription factor 2 (BHLHB2). The identification of BHLHB2 as a CAD susceptibility gene enables the development of novel therapies for CAD by screening for
10 compounds and other entities, such as antibodies, which modulate the activity of BHLHB2. Knowledge of the BHLHB2 gene sequence also enables the development of novel antigene methods to modulate the expression of the associated gene and may also enable the development of novel gene therapy techniques to treat CAD. The discovery of the linkage between BHLHB2 and CAD may also assist in developing novel methods for diagnosing
15 CAD via (i) measuring the levels of the translated mRNA of BHLHB2 present in affected tissue and (ii) measuring the levels of the BHLHB2 protein in affected tissue. It is possible that the diagnosis of CAD, or the susceptibility of an individual to CAD, by these methods may be achieved in patients who do not yet display the classical symptoms of the disease. Such determination of susceptibility to CAD, or the early detection of disease development,
20 will lead to earlier clinical intervention than is currently possible and will lead to more effective treatment of the disease.

Specifically, the invention includes a method of identifying a test compound that modulates the expression of a BHLHB2 gene, includes contacting a cell capable of expressing a
25 BHLHB2 gene with a test compound; and determining the level of expression of the BHLHB2 gene in the presence of the test compound, wherein a decrease or an increase in BHLHB2 gene expression, as compared to the level of expression of BHLHB2 in the absence of the compound, is indicative that the test compound modulates BHLHB2 gene expression.

Also within the invention is a method of identifying a test compound that modulates the activity of a protein encoded by the BHLHB2 gene, including contacting the protein with a test compound and determining the level of activity of the BHLHB2 protein in the presence of the compound, wherein a decrease or an increase in the protein activity, as compared to the level of activity of the BHLHB2 protein in the absence of the compound, is indicative that the test compound modulates BHLHB2 protein activity.

The invention further includes a method for determining if a BHLBH2 gene has an altered level of gene expression in a CAD cell. The method includes comparing the level of BHLBH2 gene expression in a cell from a patient having CAD with a control cell (a cell from a patient not having CAD), and determining the level of expression of the BHLBH2 gene in both cells, wherein a decrease or an increase in expression of the BHLBH2 gene, as compared to the level of expression of the BHLBH2 gene in the control cell, indicates that the BHLBH2 has altered gene expression.

Also within the invention is a method for determining the level of a BHLBH2 protein in a CAD patient compared to a control including comparing the protein level of BHLBH2 in a cell from a patient having CAD with a control cell (a cell from a patient not having CAD), and determining the level of the BHLBH2 protein in both cells.

In another aspect, the invention includes a method of identifying a binding partner of the BHLHB2 protein including contacting a BHLHB2 protein with a test target protein, and determining if the test target protein can interact with the BHLHB2 protein, wherein interaction of the test target protein with BHLBH2 indicates that the test target protein is a BHLBH2 binding partner. In one embodiment, the method can further include contacting a gene encoding the test target protein with a test compound; and determining the level of expression of the test target gene in the presence of the test compound, wherein a decrease or an increase in test target gene expression, as compared to the level of expression of the test target gene in the absence of the compound, is indicative that the test compound modulates expression of the test target gene and is useful in the treatment of CAD. In another

-4-

embodiment, the method further includes contacting the test target protein with a test compound; and determining the level of activity of the test target protein in the presence of the test compound, wherein a decrease or an increase in test target protein activity, as compared to the level of activity of the test target protein in the absence of the compound, is indicative that the test compound modulates test target protein activity and is useful in the treatment of CAD.

The invention further features a method of treating a subject having CAD comprising administering an effective amount of the compound identified above. The invention further includes a pharmaceutical composition comprising the compound identified as above.

In another aspect, the invention includes a method of identifying other components of the CAD biochemical pathway of which BHLHB2 is a component.

The invention further includes methods of diagnosing CAD or a susceptibility thereto in a subject. The method includes determining the level of a BHLHB2 protein in a sample from a subject; and comparing the level of the protein in the sample with a control, wherein a decrease or an increase in the level of the protein in the sample compared to the control indicates that the subject has CAD, or a susceptibility thereto. The invention also extends to products useful for carrying out the assay, such as DNA probes (labelled or unlabelled), kits and the like.

The present invention also includes gene-based therapies directed at the BHLHB2 gene. Therapies may be in the form of polynucleotides comprising all or a portion of the BHLHB2 gene, placed in appropriate vectors or delivered to target cells in direct ways which would modify the function of the BHLHB2 protein.

As used herein, a "CAD susceptibility gene" refers to a gene that has a predisposing influence on the development of CAD in a subject.

-5-

As used herein, "coronary artery disease" (CAD) refers to disorders and conditions related to the deposition of atheroma in the large- and medium-sized arteries serving the heart.

Coronary artery disease means clinical syndromes (including, but not limited to, angina, myocardial infarction, unstable angina, and sudden ischemic death) which are based on the
5 pathology of coronary artery atheroma.

As used herein "BHLHB2 protein" refers to the BHLHB2 protein, peptide fragments thereof, mutants, variants, truncated forms of BHLHB2, and fusion proteins of BHLHB2.

10

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a line graph showing the KAC LOD-score of chromosome 3.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes a CAD susceptibility gene, BHLHB2. BHLHB2, also known
15 as DEC1, is a basic helix-loop-helix transcription factor and has been implicated in control of chondrocyte (Shen-M et al. BBRC, 236:294-289, 1997), neuronal (Boudjelal, Genes and Dev 11:2052-2065, 1997) and preadipocyte differentiation (Inuzuka-H et al. BBRC 265:664-668, 1999).

The present finding shows that BHLHB2, which is mapped to chromosome region 3p, shows
20 linkage with DNA markers in CAD affected individuals. The BHLHB2 gene and amino acid sequences are known for many different species, e.g., the human BHLHB2 nucleic and amino acid sequences are available on genbank (<http://www.ncbi.nlm.nih.gov/>), under accession number AB043885 (formerly XM 005086), and the mouse BHLHB2 is available on genbank under accession number NM 011498.

25

Knowledge of the BHLHB2 sequence enables the development of novel antigene methods to modulate the expression of BHLHB2 and also enables the development of novel gene therapy techniques to treat CAD.

The sequence of human BHLHB2 protein, as disclosed in NM003670 and AB043885, is depicted in SEQ ID NO: 18, the cDNA sequence in SEQ ID NO: 19 and the genomic sequence in SEQ ID NO:20.

5 ***Functional Assay***

To further evaluate the role of BHLHB2 in CAD, various functional assays can be performed. For example techniques such as Northern analysis, *in situ* hybridization or expression profiling on cDNA microarrays can be used to further verify the association of BHLHB2 with CAD. In one example, a reporter-based assay may be devised to detect whether the BHLHB2
10 gene has a different transcription level and/or message stability compared to the same gene in a person not susceptible to CAD. Individuals who carry the BHLHB2 gene may exhibit differences in their ability to regulate protein biosynthesis under different physiological conditions and may display altered physiological abilities.

15 In another example, the level of BHLHB2 gene expression can be assayed by detecting and measuring BHLHB2 transcription. For example, RNA from a cell type or tissue known, or suspected of having CAD, can be isolated and tested utilizing hybridization or PCR techniques such as those described above. The isolated cells can be obtained from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the
20 assessment of cells to be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of BHLHB2. Such analyses can reveal both quantitative and qualitative aspects of the expression pattern of the BHLHB2 gene, including activation or inactivation of BHLHB2 gene expression.

25 In one embodiment of such a detection scheme, cDNAs are synthesized from the RNAs of interest (e.g., by reverse transcription of the RNA molecule into cDNA). A sequence within the cDNA is then used as the template for a nucleic acid amplification reaction, such as a PCR amplification reaction, or the like. For detection of the amplified product, the nucleic acid amplification may be performed using radioactively or non-radioactively labeled nucleotides.

30 Alternatively, enough amplified product can be made such that the product can be visualized

-7-

by standard ethidium bromide staining or by utilizing any other suitable nucleic acid staining method.

Additionally, it is possible to perform BHLHB2 gene expression assays *in situ*, i.e., directly
5 upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or
resections, such that no nucleic acid purification is necessary (See, e.g., Nuovo, G. J., PCR In
Situ Hybridization: Protocols And Applications, Raven Press, NY, 1992).

Alternatively, if a sufficient quantity of the appropriate cells can be obtained, standard
10 Northern analysis can be performed to determine the level of mRNA expression of the
BHLHB2 gene.

Also within the invention is the determination of whether BHLHB2 gene has a gene mutation
compared to the wild-type gene. Alternative methods for the detection of BHLHB2 gene
15 mutations in patient samples or other appropriate cell sources, may involve their
amplification, for example, by PCR (the experimental embodiment set forth in U.S. Pat. No.
4, 683, 202), followed by the detection of the amplified molecules using techniques well
known to those of skill in the art. The resulting amplified sequences can be compared to those
which would be expected if the nucleic acid being amplified contained only normal copies of
20 the BHLHB2 gene in order to determine whether a BHLHB2 gene mutation exists.

Additionally, well-known genotyping techniques can be performed to identify individuals
carrying BHLHB2 gene mutations. Such techniques include, for example, TaqMan 5'
nuclease assay, allele specific PCR, primer extension, mass spectrometry and Pyrosequencing
25 (reviewed in Kwok, Pharmacogenomics 1: 95-100, 2000)

Pathway mapping

Also within the invention is the identification and elucidation of the CAD biochemical\signal
transduction pathway of which BHLHB2 is a component. In particular, the invention includes
30 identifying the other components of the BHLHB2 CAD biochemical pathway. In this way it

is possible to identify the specific critical signaling pathway which links the disease stimulus to the cell's response and enables the identification of new potential targets for therapy intervention.

- 5 As used herein, a BHLHB2 "target molecule" is a molecule in the CAD biochemical pathway with which BHLHB2 binds or interacts, directly or indirectly, with, or is a molecule that regulates the expression of the CAD gene or translation of the protein. For example, the target molecule can be a protein which directly interacts with BHLHB2, or can be a protein which does not itself directly interact with the BHLHB2, but which is a component of the
- 10 BHLHB2 CAD biochemical pathway. In one example, the target molecule can be a cell membrane or a cytoplasmic molecule. In another example, the target molecule can be an intercellular protein or a protein which facilitates the association of downstream or upstream signaling molecules with the BHLHB2 protein.
- 15 According to a further aspect of the invention there is provided the use of the BHLHB2 protein in research to identify further gene targets implicated in CAD. Methods for identifying proteins which interact with BHLHB2 are known in the art, e.g., the two-hybrid assay (see, e.g., U.S. Pat. No. 5,283,317; Zervos et al. Cell 72:223-232, 1993) or using cell culture techniques to identify binding partners.
- 20 The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for a BHLHB2 protein is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g.,
- 25 GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo*, forming a BHLHB2-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity
- 30 allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter

gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the "target" protein which interacts with the BHLHB2 protein.

- 5 Alternatively, binding partners for BHLHB2 can be identified using cell culture techniques or using cells obtained directly from a CAD patient. The method includes isolating the BHLHB2 protein from the cell and determining the identity of its target molecule. Initial screening can be accomplished by Western blot analysis to analyse immunochemically, e.g., using antibodies against the BHLHB2, the size of BHLHB2 -target molecule complex.
- 10 Further analysis of the complex will reveal the identity of the target molecule.

- The gene and protein encoded by the target molecule is also a potential target for therapeutic intervention in CAD disease, for instance in the development of antisense nucleic acid targeted to the mRNA; or more widely in the identification or development of chemical or
- 15 hormonal therapeutic agents. The person skilled in the art is also capable of devising screening assays to identify compounds (chemical or biological) that modulate (activate or inhibit) the identified gene, which compounds may prove useful as therapeutic agents in treating or preventing CAD.

20 *BHLHB2 Proteins and Polypeptides*

- BHLHB2 proteins, peptide fragments, mutated, and truncated forms of the BHLHB2, and/or BHLHB2 fusion proteins, can be prepared for a variety of uses, including but not limited to the generation of antibodies, as reagents in diagnostic assays, in the identification of other cellular gene products involved in the pathogenesis of CAD, as reagents in assays for
- 25 screening for compounds that can be used in the treatment of CAD, and as pharmaceutical reagents useful in the treatment of CAD.

- A variety of host-expression vector systems may be utilized to express the BHLHB2 nucleotide sequences of the invention. The expression systems that may be used for purposes
- 30 of the invention include but are not limited to microorganisms such as bacteria (e.g., *E. coli*,

B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing BHLHB2 nucleotide sequences or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harbouring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or
5 from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the BHLHB2 gene product being expressed. For
10 example, when a large quantity of BHLHB2 is to be produced, for the generation of pharmaceutical compositions of BHLHB2 or for raising antibodies to the BHLHB2 protein, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther et al., EMBO J. 2:1791, 1983), in which the BHLHB2
15 coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, Nucleic Acids Res. 13:3101-3109, 1985; Van Heeke & Schuster, J. Biol. Chem. 264:5503-5509, 1989); and the like. PGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be
20 purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

25 In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the BHLHB2 nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of
30 the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and

-11-

capable of expressing the BHLHB2 in infected hosts. (E.g., See Logan & Shenk, , Proc. Natl. Acad. Sci. USA 81:3655-3659, 1984).

For long-term, high-yield production of recombinant proteins, stable expression is preferred.

5 For example, cell lines which stably express the BHLHB2 sequence described above may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered

10 cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the BHLHB2. Such engineered

15 cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the BHLHB2.

Alternatively, any fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al.

20 allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., Proc. Natl. Acad. Sci. USA 88:8972-8976, 1991). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni.2+

25 nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

The BHLHB2 protein can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-

30 human primates, for example, baboons, monkeys, and chimpanzees may be used to generate

-12-

BHLHB2 transgenic animals.

Any technique known in the art may be used to introduce the BHLHB2 transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not
5 limited to pronuclear microinjection (U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci. USA 82:6148-6152, 1985); gene targeting in embryonic stem cells (Thompson et al., Cell 56:313-321, 1989); electroporation of embryos (Lo, Mol. Cell. Biol. 3:1803-1814, 1983); and sperm-mediated gene transfer (Lavitrano et al., Cell 57:717-723, 1989); etc. For a review of such techniques,
10 see Gordon, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, 1989, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the BHLHB2 transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e.,
15 mosaic animals. The transgene may be integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko, M. et al., Proc. Natl. Acad. Sci. USA 89:6232-6236, 1992). Once transgenic animals have been generated, the expression of the recombinant BHLHB2 gene
20 may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include but are not limited to Northern blot analysis of tissue samples obtained from the animal, in
25 situ hybridization analysis, and RT-PCR. Samples of BHLHB2 gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the BHLHB2 transgene product.

Antibodies to BHLHB2 Proteins

30 Antibodies that specifically recognize one or more epitopes of BHLHB2 or peptide fragments

-13-

of the BHLHB2 are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention may be used, for example, in the detection of the BHLHB2 in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of BHLHB2. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of the BHLHB2 gene product. Additionally, such antibodies can be used in conjunction with the gene therapy techniques described, for example, evaluate the normal and/or engineered BHLHB2-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition of abnormal BHLHB2 activity. Thus, such antibodies may, therefore, be utilized as part of CAD treatment methods.

Methods of making and detecting labelled antibodies are well known (Campbell; Monoclonal Antibody Technology, in: Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13. Eds: Burdon R et al. Elsevier, Amsterdam (1984)). The term antibody includes both monoclonal antibodies, which are a substantially homogeneous population, and polyclonal antibodies which are heterogeneous populations. The term also includes inter alia, humanised and chimeric antibodies. Monoclonal antibodies to specific antigens may be obtained by methods known to those skilled in the art, such as from hybridoma cells, phage display libraries or other methods. Monoclonal antibodies may be inter alia, human, rat or mouse derived. For the production of human monoclonal antibodies, hybridoma cells may be prepared by fusing spleen cells from an immunised animal, e.g. a mouse, with a tumour cell. Appropriately secreting hybridoma cells may thereafter be selected (Koehler & Milstein, Nature 256:495-497 (1975); Cole et al., "Monoclonal antibodies and Cancer Therapy", Alan

R Liss Inc, New York N.Y. pp 77-96 (1985)). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof.

Polyclonal antibodies can be generated by immunisation of an animal (such as a mouse, rat,
5 goat, horse, sheep etc) with BHLHB2.

Rodent antibodies may be humanised using recombinant DNA technology according to techniques known in the art. Alternatively, chimeric antibodies, single chain antibodies, Fab fragments may also be developed against the polypeptides of the invention (Huse et al.,
10 Science 256:1275-1281 (1989)), using skills known in the art. Antibodies so produced have a number of uses which will be evident to the molecular biologist or immunologist skilled in the art. Such uses include, but are not limited to, monitoring enzyme expression, development of assays to measure enzyme activity and use as a therapeutic agent. Enzyme linked immunosorbant assays (ELISAs) are well known in the art and would be particularly suitable
15 for detecting the BHLHB2 or polypeptide fragments thereof in a test sample.

Screening Assay

The invention also provides a method for identifying modulators, i.e., test compounds (e.g., peptides, peptidomimetics, small molecules or other drugs) which modulate the activity of the
20 BHLHB2 gene or protein.

In one example, the invention provides methods for screening for test compounds for use in the treatment of CAD by screening for test compounds that modulate the activity of the BHLHB2 protein, or a portion thereof. The test compounds of the present invention can be
25 obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries. The method can be a cell-based method or a cell free method. The screening methods according to the invention may be operated using conventional procedures, for example by bringing the test compound or compounds to be screened and an appropriate substrate into contact with the BHLHB2 polypeptide, or a cell
30 capable of producing it, or a cell membrane preparation thereof, and determining affinity for the BHLHB2 polypeptide in accordance with standard techniques.

-15-

Any compound identified in this way may prove useful in the treatment of CAD in humans and/or other animals. The invention thus extends to a compound selected through its ability to regulate the activity of the BHLHB2 protein *in vivo* as primarily determined in a screening assay utilising a BHLHB2 polypeptide or a homologue or fragment thereof, or a gene coding therefore for use in the treatment of a disease in which the over- or under-activity or unregulated activity of the protein is implicated.

According to a further aspect of the invention there is provided a screening assay or method for identifying potential CAD therapeutic compounds comprising contacting an assay system capable of detecting the effect of a test compound against expression level of BHLHB2, with a test compound and assessing the change in expression level of BHLHB2. Compounds that modulate the expression of DNA or RNA of the BHLHB2 polypeptide may be detected by a variety of assay systems. A suitable assay system may be a simple "yes/no" assay to determine whether there is a change in expression of a reporter gene, such as beta-galactosidase, luciferase, green fluorescent protein or others known to the person skilled in the art (reviewed by Naylor, Biochem. Pharmacol. 58:749-57, 1999). The assay system may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample. Systems in which transcription factors are used to stimulate a positive output, such as transcription of a reporter gene, are generally referred to as "one-hybrid systems" (Wang, M.M. and Reed, R.R. Nature 364:121-126, 1993). Using a transcription factor to stimulate a negative output (growth inhibition) may thus be referred to as a "reverse one-hybrid system" (Vidal et al, 1996, *supra*). Therefore, in an embodiment of the present invention, a reporter gene is placed under the control of the BHLHB2 promoter.

25 In a further aspect of the invention a cell or cell line comprising a reporter gene under the control of the BHLHB2 promoter is provided.

According to another aspect of the present invention there is provided a method of screening for a compound potentially useful for treatment of CAD, which comprises assaying the compound for its ability to modulate the activity or amount of BHLHB2. Preferably the assay is selected from:

-16-

- i) measurement of BHLHB2 activity using a cell line which expresses the BHLHB2 polypeptide or using purified BHLHB2 polypeptide; and
- ii) measurement of BHLHB2 transcription or translation in a cell line expressing the BHLHB2 polypeptide.

5

Thus, in a further aspect of the invention, cell cultures expressing the BHLHB2 polypeptide can be used in a screen for therapeutic agents. Effects of test compounds may be assayed by changes in mRNA or protein of BHLHB2. As described above, cells (i.e. mammalian, bacterial, etc) can be engineered to express the BHLHB2 polypeptide.

10

Thus, according to a further aspect of the invention there is provided a method of testing potential therapeutic agents for the ability to suppress the CAD phenotype comprising contacting a test compound with a cell engineered to express the BHLHB2 polypeptide; and determining whether said test compound suppressed expression of the BHLHB2 polypeptide.

15

We also provide a method for identifying inhibitors of transcription of BHLHB2, which method comprises contacting a potential therapeutic agent with a cell or cell line as described above and determining inhibition of BHLHB2 transcription by the potential therapeutic agent by reference to a lack of or reduction in expression of the reporter gene.

20

Any convenient test compound or library of test compounds may be used in conjunction with the test assay. Particular test compounds include low molecular weight chemical compounds (preferably with a molecular weight less than 1500 daltons) suitable as pharmaceutical or veterinary agents for human or animal use, or compounds for non-administered use such as cleaning/sterilising agents or for agricultural use. Test compounds may also be biological in nature, such as antibodies.

25

According to a further aspect of the invention there is provided a compound identified by a screening method as defined herein.

30

According to another aspect of the present invention there is provided use of a compound able to modulate the activity or amount of BHLHB2 in the preparation of a medicament for the

treatment of CAD. Modulation of the amount of BHLHB2 by a compound may be brought about for example through altered gene expression level or message stability. Modulation of the activity of BHLHB2 by a compound may also be brought about for example through compound binding to the BHLHB2 protein. In one embodiment, modulation of BHLHB2
5 comprises use of a compound able to reduce the activity or amount of BHLHB2. In another embodiment, modulation of BHLHB2 comprises use of a compound able to increase the activity or amount of BHLHB2.

Diagnostic test

10 Methods for the diagnosis of CAD may, for example, utilize reagents such as the BHLHB2 nucleotide sequences and BHLHB2 antibodies. Specifically, such reagents may be used, for example, for: (1) the detection of the presence of BHLHB2 gene mutations, or the detection of either over- or under-expression of BHLHB2 mRNA relative to a control; (2) the detection of either an over- or an under-abundance of BHLHB2 gene product relative to a control; and (3)
15 the detection of perturbations or abnormalities in the signal transduction pathway mediated by BHLHB2.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific BHLHB2 nucleotide sequence or BHLHB2
20 antibody reagent described herein, which may be conveniently used, e.g., in clinical settings, to diagnose patients exhibiting body weight disorder abnormalities.

For the detection of BHLHB2 mutations, any nucleated cell can be used as a starting source for genomic nucleic acid. For the detection of BHLHB2 gene expression or BHLHB2 gene
25 products, any cell type or tissue in which the BHLHB2 gene is expressed can be used.

Detection of BHLHB2 polymorphisms

Knowledge of polymorphisms can be of assistance in identifying patients susceptible to
30 particular diseases and those most suited to therapy with particular pharmaceutical agents (the latter is often termed "pharmacogenetics"). Pharmacogenetics can also be used in

-18-

pharmaceutical research to assist the drug selection process. Polymorphisms are used in mapping the human genome and to elucidate the genetic component of diseases. The reader is directed to the following references for background details on pharmacogenetics and other uses of polymorphism detection: Linder *et al.* (1997), *Clinical Chemistry*, 43, 254; Marshall
5 (1997), *Nature Biotechnology*, 15, 1249; International Patent Application WO 97/40462, Spectra Biomedical; and Schafer *et al.* (1998), *Nature Biotechnology*, 16, 33.

The present inventors have identified a number of polymorphisms within and around the BHLHB2 gene with demonstrable genetic association to coronary artery disease (CAD). The
10 type and position of polymorphisms, which are identified in Table 3, are identified according to the position in Genbank ID:AB043885. Tables 4 and 5 provide sequence adjacent to the polymorphism site, which sequence can be used to unambiguously locate the position of the polymorphism in a nucleic acid sample, without reference to AB043885. SEQ ID Nos: 1 – 17 provide sequence of the polymorphism and sequence adjacent the polymorphism.

15 According to one aspect of the present invention there is provided a method for the diagnosis of a nucleotide polymorphism associated with CAD, which method comprises determining the sequence of the nucleotide at position 61 of any one of SEQ ID NOs: 1 to 5, 7, 8 and 10 to 17, or determining the number of CA repeats starting at position 61 of SEQ ID NO: 6, or the
20 number of GT repeats starting at position 61 of SEQ ID NO: 9, and determining the status of the human by reference to polymorphism(s) detected.

The term human includes both a human having or suspected of having inflammatory bowel disease and an asymptomatic human who may be tested for predisposition or susceptibility to
25 CAD. At each position the human may be homozygous for an allele or the human may be a heterozygote.

In one embodiment of the invention preferably the method for diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 1)
30 is the presence of G and/or A.

-19-

In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 2) is the presence of A and/or C.

- 5 In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 3) is the presence of C and/or T.

- In another embodiment of the invention preferably the method of diagnosis described herein
10 is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 4) is the presence of C and/or T.

- In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO:
15 5) is the presence of C and/or T.

- In another embodiment of the invention preferably the method of diagnosis described herein is one in which the number of CA repeats starting at position 61 (according to SEQ ID NO: 6) is determined.

20

- In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 7) is the presence of T and/or C.

- 25 In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 8) is the presence of A and/or G.

- In another embodiment of the invention preferably the method of diagnosis described herein
30 is one in which the number of GT repeats starting at position 61 (according to SEQ ID NO: 9) is determined.

-20-

In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 10) is the presence of G and/or C.

- 5 In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 11) is the presence of G and/or T.

- 10 In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 12) is the presence of G and/or A.

- In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 15 13) is the presence of A and/or G.

- In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 14) is the presence of A and/or T.

20

- In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 15) is the presence of A and/or C.

- 25 In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 16) is the presence of G and/or A.

- In another embodiment of the invention preferably the method of diagnosis described herein 30 is one in which the single nucleotide polymorphism at position 61 (according to SEQ ID NO: 17) is the presence of A and/or T.

-21-

The method for diagnosis is preferably one in which the sequence is determined by a method selected from amplification refractory mutation system and restriction fragment length polymorphism.

- 5 In another aspect of the invention we provide a method for the diagnosis or prognosis or inflammatory bowel disease, which method comprises:
- i) obtaining sample nucleic acid from an individual,
 - ii) detecting the presence or absence of a variant nucleotide at position 61 relative to one or more of the sequences disclosed in SEQ ID NO: 1 to 5, 7, 8 and 10 to 17, or detecting the
10 number of CA repeats starting at position 61 of SEQ ID NO: 6, or the number of GT repeats starting at position 61 of SEQ ID NO: 9; and,
 - iii) determining the status of the individual by reference to the particular variant polymorphism.
- 15 The test sample of nucleic acid is conveniently a sample of blood, bronchoalveolar lavage fluid, sputum, or other body fluid or tissue obtained from an individual. It will be appreciated that the test sample may equally be a nucleic acid sequence corresponding to the sequence in the test sample, that is to say that all or a part of the region in the sample nucleic acid may firstly be amplified using any convenient technique e.g. PCR, before use in the analysis of
20 BHLHB2 variation.

It will be apparent to the person skilled in the art that there are a large number of analytical procedures, which may be used to detect the presence or absence of the various SNPs of the invention. In general, the detection of allelic variation requires a mutation discrimination
25 technique, optionally an amplification reaction and a signal generation system. Table 1 lists a number of mutation detection techniques, some based on the PCR. These may be used in combination with a number of signal generation systems, a selection of which is listed in List 1. Further amplification techniques are listed in List 2. Many current methods for the detection of allelic variation are reviewed by Nollau *et al.*, Clin. Chem. 43, 1114-1120, 1997;
30 and in standard textbooks, for example "Laboratory Protocols for Mutation Detection", Ed. by U. Landegren, Oxford University Press, 1996 and "PCR", 2nd Edition by Newton & Graham, BIOS Scientific Publishers Limited, 1997.

Abbreviations:

ALEX™	Amplification refractory mutation system linear extension
APEX	Arrayed primer extension
ARMST™	Amplification refractory mutation system
b-DNA	Branched DNA
CMC	Chemical mismatch cleavage
Bp	base pair
COPS	Competitive oligonucleotide priming system
DGGE	Denaturing gradient gel electrophoresis
FRET	Fluorescence resonance energy transfer
LCR	Ligase chain reaction
MASDA	Multiple allele specific diagnostic assay
NASBA	Nucleic acid sequence based amplification
OLA	Oligonucleotide ligation assay
PCR	Polymerase chain reaction
PTT	Protein truncation test
RFLP	Restriction fragment length polymorphism
SDA	Strand displacement amplification
SNP	Single nucleotide polymorphism
SSCP	Single-strand conformation polymorphism analysis
SSR	Self sustained replication
TGGE	Temperature gradient gel electrophoresis

Table 1 - Mutation Detection Techniques**General:** DNA sequencing, Sequencing by hybridisation

- 5 **Scanning:** PTT*, SSCP, DGGE, TGGE, Cleavase, Heteroduplex analysis, CMC, Enzymatic mismatch cleavage

* Note: not useful for detection of promoter polymorphisms.

Hybridisation Based

Solid phase hybridisation: Dot blots, MASDA, Reverse dot blots, Oligonucleotide arrays (DNA Chips)

Solution phase hybridisation: Taqman™ - US-5210015 & US-5487972 (Hoffmann-La Roche), Molecular Beacons - Tyagi *et al* (1996), Nature Biotechnology, 14, 303; WO 95/13399 (Public Health Inst., New York)

Extension Based: ARMST™, ALEX™ - European Patent No. EP 332435 B1 (Zeneca Limited), COPS - Gibbs *et al* (1989), Nucleic Acids Research, 17, 2347.

Incorporation Based: Mini-sequencing, APEX

10 Restriction Enzyme Based: RFLP, Restriction site generating PCR

Ligation Based: OLA

Other: Invader assay

List 1 - Signal Generation or Detection Systems

15 Fluorescence: FRET, Fluorescence quenching, Fluorescence polarisation - United Kingdom Patent No. 2228998 (Zeneca Limited)

Other: Chemiluminescence, Electrochemiluminescence, Raman, Radioactivity, Colorimetric, Hybridisation protection assay, Mass spectrometry

20 **List 2 - Further Amplification Methods**

SSR, NASBA, LCR, SDA, b-DNA

Preferred mutation detection techniques include ARMST™, ALEX™, COPS, Taqman, Molecular Beacons, RFLP, and restriction site based PCR and FRET techniques.

25

Particularly preferred methods include Taqman, ARMST™ and RFLP based methods. Taqman is an especially preferred method.

Assays, for example reporter-based assays, may be devised to detect whether one or more of
30 the above polymorphisms affect transcription levels.

In a further aspect, the diagnostic methods of the invention, are used to assess the predisposition and/or susceptibility of an individual to CAD, and the present invention may be used to recognise individuals who are particularly at risk from developing CAD conditions.

- 5 In a further aspect, the diagnostic methods of the invention are used in the development of new drug therapies, which selectively target one or more allelic variants identified herein. Identification of a link between a particular allelic variant and predisposition to disease development or response to drug therapy may have a significant impact on the design of new drugs. Drugs may be designed to regulate the biological activity of variants implicated in the
10 disease process whilst minimising effects on other variants.

In a further diagnostic aspect of the invention the presence or absence of variant nucleotides is detected by reference to the loss or gain of, optionally engineered, sites recognised by restriction enzymes. The person of ordinary skill will be able to design and implement
15 diagnostic procedures based on the detection of restriction fragment length polymorphism due to the loss or gain of one or more of the sites.

The invention further provides nucleotide primers which detect the polymorphisms of the invention.

20

The invention further provides nucleotide probes which can detect the polymorphisms of the invention.

According to another aspect of the present invention a person of ordinary skill will be able to
25 design allele specific primers or probes capable of detecting a polymorphism at position 61 at one or more of: SEQ ID NO: 1 to 17.

An allele specific primer is used, generally together with a constant primer, in an amplification reaction such as a PCR reaction, which provides the discrimination between
30 alleles through selective amplification of one allele at a particular sequence position e.g. as used for ARMS™ assays. The allele specific primer is preferably 17- 50 nucleotides, more preferably about 17-35 nucleotides, more preferably about 17-30 nucleotides.

-25-

An allele specific primer preferably corresponds exactly with the allele to be detected but derivatives thereof are also contemplated wherein about 6-8 of the nucleotides at the 3' terminus correspond with the allele to be detected and wherein up to 10, such as up to 8, 6, 4, 2, or 1 of the remaining nucleotides may be varied without significantly affecting the
5 properties of the primer.

Primers may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example "Protocols for Oligonucleotides and Analogues; Synthesis and Properties," Methods in Molecular Biology Series; Volume 20;
10 Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition. If required the primer(s) may be labelled to facilitate detection.

According to another aspect of the present invention there is provided an allele-specific oligonucleotide probe capable of detecting a polymorphism at position 61 of any one of SEQ
15 ID NOs: 1 to 5, 7, 8 and 10 to 17, or a variant number of CA repeats starting at position 61 of SEQ ID NO: 6, or a variant number of GT repeats starting at position 61 of SEQ ID NO: 9.

The allele-specific oligonucleotide probe is preferably 17- 50 nucleotides, more preferably about 17-35 nucleotides, more preferably about 17-30 nucleotides. Representative examples
20 of oligonucleotide probes are those identified in SEQ ID NO: 1 to 17, but wherein the nucleotide at position 31 is one or other of the polymorphic alleles.

The design of such probes will be apparent to the molecular biologist of ordinary skill. Such probes are of any convenient length such as up to 50 bases, up to 40 bases, more conveniently
25 up to 30 bases in length, such as for example 8-25 or 8-15 bases in length. In general such probes will comprise base sequences entirely complementary to the corresponding wild type or variant locus in the gene. However, if required one or more mismatches may be introduced, provided that the discriminatory power of the oligonucleotide probe is not unduly affected. The probes of the invention may carry one or more labels to facilitate detection.
30 According to another aspect of the present invention there is provided a diagnostic kit comprising a diagnostic primer of the invention and/or an allele-specific oligonucleotide primer of the invention.

-26-

The diagnostic kits may comprise appropriate packaging and instructions for use in the methods of the invention. Such kits may further comprise appropriate buffer(s) and polymerase(s) such as thermostable polymerases, for example taq polymerase.

- 5 In another aspect of the invention, the single nucleotide polymorphisms of this invention may be used as genetic markers for this region in linkage studies.

Detection of the CAD Gene and Transcripts

- BHLHB2 gene transcripts and mutations within the BHLHB2 gene can be detected by
10 utilizing a number of techniques. Nucleic acid from any nucleated cell can be used as the starting point for such assay techniques, and may be isolated according to standard nucleic acid preparation procedures which are well known to those of skill in the art.

- DNA may be used in hybridization or amplification assays of biological samples to detect
15 abnormalities involving BHLHB2 gene structure or gene regulatory elements, including point mutations, insertions, deletions and chromosomal rearrangements. Such assays may include, but are not limited to, Southern analyses, single stranded conformational polymorphism analyses (SSCP), and PCR analyses.

- 20 Such diagnostic methods for the detection of BHLHB2 gene-specific mutations can involve for example, contacting and incubating nucleic acids including recombinant DNA molecules, cloned genes or degenerate variants thereof, obtained from a sample, for example, derived from a patient sample or other appropriate cellular source, with one or more labeled nucleic acid reagents including recombinant DNA molecules, under conditions favorable for the
25 specific annealing of these reagents to their complementary sequences within the BHLHB2 gene. Preferably, the lengths of these nucleic acid reagents are at least 15 to 30 nucleotides. After incubation, all non-annealed nucleic acids are removed from the nucleic acid: BHLHB2 molecule hybrid. The presence of nucleic acids which have hybridized, if any such molecules exist, is then detected. Using such a detection scheme, the nucleic acid from the cell type or
30 tissue of interest can be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtiter plate or polystyrene beads. Detection of the remaining, annealed, labeled BHLHB2 nucleic acid reagents is accomplished using standard

-27-

techniques well-known to those in the art. The BHLHB2 gene sequences to which the nucleic acid reagents have annealed can be compared to the annealing pattern expected from a normal BHLHB2 gene sequence in order to determine whether a BHLHB2 gene mutation is present.

5 *Detection of the BHLHB2 Gene Products*

Antibodies directed against wild type or mutant BHLHB2 gene products or conserved variants or peptide fragments thereof, can also be used as CAD diagnostics. Such diagnostic methods, can be used to detect abnormalities in the level of BHLHB2 gene expression, or abnormalities in the structure and/or temporal, tissue, cellular, or subcellular location of the
10 BHLHB2, and may be performed in vivo or in vitro, such as, for example, on biopsy tissue.

For example, antibodies directed to epitopes of the BHLHB2 can be used in vivo to detect the pattern and level of expression of the BHLHB2 in the body. Such antibodies can be labeled, and injected into a subject in order to visualize binding to the BHLHB2 expressed in the body
15 using methods such as X-rays, CAT-scans, or MRI.

Additionally, any BHLHB2 fusion protein or BHLHB2 conjugated protein whose presence can be detected, can be administered. For example, BHLHB2 fusion or conjugated proteins labeled with a radio-opaque or other appropriate compound can be administered and
20 visualized in vivo, as discussed, above for labeled antibodies.

Alternatively, immunoassays or fusion protein detection assays, as described above, can be utilized on biopsy and autopsy samples in vitro to permit assessment of the expression pattern of the BHLHB2.

25 The tissue or cell type to be analyzed will generally include those which are known, or suspected, to express the BHLHB2 gene. The protein isolation methods employed herein can, for example, be such as those described in Harlow and Lane (Harlow, E. and Lane, D, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring
30 Harbor, N.Y., 1989), which is incorporated herein by reference in its entirety. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from

culture may be a necessary step in the assessment of cells that could be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the BHLHB2 gene.

5 *Gene therapy*

The invention encompasses gene therapy methods and compositions for treating and preventing CAD.

10 In one example, the loss of normal BHLHB2 gene product function can result in the development of CAD. Increasing BHLHB2 gene product activity, or activation of the BHLHB2 pathway (e.g., downstream activation) would therefore facilitate progress in individuals exhibiting a deficient level of BHLHB2 gene expression and/or BHLHB2 activity.

15 Alternatively, CAD may be ameliorated by decreasing the level of BHLHB2 gene expression, and/or BHLHB2 gene activity, and/or downregulating activity of the BHLHB2 pathway (e.g., by targeting downstream signaling events). Different approaches are discussed below.

Inhibition of BHLHB2 Expression or BHLHB2 Activity

20 Any method that neutralizes or inhibits expression of the BHLHB2 gene (either transcription or translation) can be used to prevent or treat CAD.

For example, the administration of soluble peptides, proteins, fusion proteins, or antibodies (including anti-idiotypic antibodies) that bind to and "neutralize" circulating BHLHB2 can be used. Such BHLHB2 neutralizing peptides, proteins, fusion proteins, anti-idiotypic antibodies
25 or Fabs are administered to a subject in amounts sufficient to treat or prevent CAD.

In an alternate embodiment, therapy can be designed to reduce the level of endogenous BHLHB2 gene expression, for example, using antisense or ribozyme approaches to inhibit or prevent translation of BHLHB2 mRNA-transcripts; triple helix approaches to inhibit
30 transcription of the BHLHB2 gene; or targeted homologous recombination to inactivate or

"knock out" the BHLHB2 gene or its endogenous promoter. Antisense, ribozyme or DNA constructs can be administered directly to the site containing the target cells; or can be directed to the target cells.

- 5 Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to BHLHB2 mRNA. The antisense oligonucleotides will bind to the complementary BHLHB2 mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity
- 10 to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still
- 15 form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

- Oligonucleotides that are complementary to the 5' end of the message, for example, the 5'
- 20 untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. Thus, oligonucleotides complementary to either the 5'- or 3'-non-translated, non-coding regions of the BHLHB2 can be used in an antisense approach to inhibit translation of
- 25 endogenous BHLHB2 mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5'-, 3'- or coding region of BHLHB2 mRNA, antisense nucleic acids should be at least
- 30 six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50

nucleotides in length. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides, or at least 50 nucleotides in length.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

While antisense nucleotides complementary to the BHLHB2 coding region sequence could be used, those complementary to the transcribed untranslated region are most preferred. The antisense molecules should be delivered to cells which express the BHLHB2 *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells; for example, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (e.g., antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

Ribozyme molecules designed to catalytically cleave BHLHB2 mRNA transcripts can also be used to prevent translation of BHLHB2 mRNA and expression of BHLHB2. (See, e.g., WO90/11364; Sarver et al., Science 247:1222-1225, 1990). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy BHLHB2 mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two

-31-

bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, Nature 334:585-591, 1988. There are hundreds of potential hammerhead ribozyme cleavage sites within the nucleotide sequence of human BHLHB2 cDNA. Preferably the ribozyme is engineered so that the cleavage
5 recognition site is located near the 5' end of the BHLHB2 mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in Tetrahymena Thermophila
10 (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, et al., Science 224:574-578, 1984; Zaug and Cech, Science 231:470-475, 1986; Zaug, et al., Nature 324:429-433, 1986; patent application No. WO 88/04300; Been and Cech, Cell 47:207-216, 1986). The Cech-type ribozymes have an eight basepair active site that hybridizes to a target RNA sequence, whereafter cleavage of the
15 target RNA takes place. The invention encompasses those Cech-type ribozymes that target eight basepair active site sequences that are present in BHLHB2.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express the
20 BHLHB2 in vivo. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous BHLHB2 messages and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

25 In an alternative embodiment for neutralizing circulating BHLHB2, cells that are genetically engineered to express such soluble or secreted forms of BHLHB2 can be administered to a patient, whereupon they will serve as "bioreactors" in vivo to provide a continuous supply of the neutralizing protein.
30

-32-

Endogenous BHLHB2 gene expression can also be reduced by inactivating or "knocking out" the BHLHB2 gene or its promoter using targeted homologous recombination. (E.g., see Smithies et al., Nature 317:230-234, 1985; Thomas & Capecchi, Cell 51:503-512, 1987; Thompson et al., Cell 5:313-321, 1989; each of which is incorporated by reference herein in its entirety).

In yet another embodiment of the invention, the activity of BHLHB2 can be reduced using a "dominant negative" approach to effectuate BHLHB2. To this end, constructs that encode defective BHLHB2s can be used in gene therapy approaches to diminish the activity of the BHLHB2 in appropriate target cells.

Restoration or Increase in BHLHB2 Expression or Activity

With respect to an increase in the level of BHLHB2 gene expression and/or BHLHB2 gene product activity, BHLHB2 nucleic acid sequences can be utilized for the treatment of CAD. Where the cause of CAD is a defective BHLHB2 gene, treatment can be administered, for example, in the form of gene replacement therapy. Specifically, one or more copies of a normal BHLHB2 gene or a portion of the BHLHB2 gene that directs the production of a BHLHB2 gene product exhibiting normal function, can be inserted into the appropriate cells within a patient or animal subject, using vectors which include, but are not limited to, adenovirus, adeno-associated virus, retrovirus, and herpes virus vectors, in addition to other particles that introduce DNA into cells, such as liposomes.

Additional methods that can be utilized to increase the overall level of BHLHB2 gene expression and/or BHLHB2 activity include the introduction of appropriate BHLHB2-expressing cells, preferably autologous cells, into a patient at positions and in numbers which are sufficient to ameliorate the symptoms of CAD. Such cells can be either recombinant or non-recombinant. Among the cells that can be administered to increase the overall level of BHLHB2 gene expression in a patient are normal cells which express the BHLHB2 gene. The cells can be administered to the site of interest. Such cell-based gene therapy techniques are well known to those skilled in the art, see, for example, Anderson, et al., U.S. Pat. No. 5,399,349; Mulligan & Wilson, U.S. Pat. No. 5,460,959.

Finally, compounds, identified in the assays described above, that stimulate or enhance the signal transduced by activated BHLHB2, for example, by activating downstream signalling proteins in the BHLHB2 cascade, and thereby by-passing the defective BHLHB2. The formulation and mode of administration will depend upon the physico-chemical properties of the compound.

Pharmaceutical composition containing the Identified Candidate Compound

The compounds having the desired activity may be administered in a physiologically acceptable carrier to a CAD patient. Such compositions of the invention may be in a form suitable for oral use (for example as tablets, lozenges, hard or soft capsules, aqueous or oily suspensions, emulsions, dispersible powders or granules, syrups or elixirs), for topical use (for example as creams, ointments, gels, or aqueous or oily solutions or suspensions), for administration by inhalation (for example as a finely divided powder or a liquid aerosol), for administration by insufflation (for example as a finely divided powder) or for parenteral administration (for example as a sterile aqueous or oily solution for intravenous, subcutaneous, intramuscular or intramuscular dosing or as a suppository for rectal dosing).

The compositions of the invention may be obtained by conventional procedures using conventional pharmaceutical excipients, well known in the art. Thus, compositions intended for oral use may contain, for example, one or more colouring, sweetening, flavouring and/or preservative agents.

Suitable pharmaceutically acceptable excipients for a tablet formulation include, for example, inert diluents such as lactose, sodium carbonate, calcium phosphate or calcium carbonate, granulating and disintegrating agents such as corn starch or algenic acid; binding agents such as starch; lubricating agents such as magnesium stearate, stearic acid or talc; preservative agents such as ethyl or propyl p-hydroxybenzoate, and anti-oxidants, such as ascorbic acid. Tablet formulations may be uncoated or coated either to modify their disintegration and the subsequent absorption of the active ingredient within the gastrointestinal track, or to improve their stability and/or appearance, in either case, using conventional coating agents and procedures well known in the art.

Compositions for oral use may be in the form of hard gelatin capsules in which the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules in which the active ingredient is mixed with water or an oil such as peanut oil, liquid paraffin, or olive oil.

5

Aqueous suspensions generally contain the active ingredient in finely powdered form together with one or more suspending agents, such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinyl-pyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents such as lecithin or condensation
10 products of an alkylene oxide with fatty acids (for example polyoxyethylene stearate), or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example
15 heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives (such as ethyl or propyl p-hydroxybenzoate, anti-
20 oxidants (such as ascorbic acid), colouring agents, flavouring agents, and/or sweetening agents (such as sucrose, saccharine or aspartame).

Oily suspensions may be formulated by suspending the active ingredient in a vegetable oil (such as arachis oil, olive oil, sesame oil or coconut oil) or in a mineral oil (such as liquid
25 paraffin). The oily suspensions may also contain a thickening agent such as beeswax, hard paraffin or cetyl alcohol. Sweetening agents such as those set out above, and flavouring agents may be added to provide a palatable oral preparation. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid.

30 Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water generally contain the active ingredient together with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents

and suspending agents are exemplified by those already mentioned above. Additional excipients such as sweetening, flavouring and colouring agents, may also be present.

The pharmaceutical compositions of the invention may also be in the form of

5 oil-in-water emulsions. The oily phase may be a vegetable oil, such as olive oil or arachis oil, or a mineral oil, such as for example liquid paraffin or a mixture of any of these. Suitable emulsifying agents may be, for example, naturally-occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soya bean, lecithin, an esters or partial esters derived from fatty acids and hexitol anhydrides (for example sorbitan monooleate) and

10 condensation products of the said partial esters with ethylene oxide such as polyoxyethylene sorbitan monooleate. The emulsions may also contain sweetening, flavouring and preservative agents.

Syrups and elixirs may be formulated with sweetening agents such as glycerol, propylene

15 glycol, sorbitol, aspartame or sucrose, and may also contain a demulcent, preservative, flavouring and/or colouring agent.

The pharmaceutical compositions may also be in the form of a sterile injectable aqueous or oily suspension, which may be formulated according to known procedures using one or more

20 of the appropriate dispersing or wetting agents and suspending agents, which have been mentioned above. A sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example a solution in 1,3-butanediol.

25 Suppository formulations may be prepared by mixing the active ingredient with a suitable non-irritating excipient which is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Suitable excipients include, for example, cocoa butter and polyethylene glycols.

30 Topical formulations, such as creams, ointments, gels and aqueous or oily solutions or suspensions, may generally be obtained by formulating an active ingredient with a

-36-

conventional, topically acceptable, vehicle or diluent using conventional procedure well known in the art.

Compositions for administration by insufflation may be in the form of a finely divided powder containing particles of average diameter of, for example, 30μ or much less, the powder itself comprising either active ingredient alone or diluted with one or more physiologically acceptable carriers such as lactose. The powder for insufflation is then conveniently retained in a capsule containing, for example, 1 to 50mg of active ingredient for use with a turbo-inhaler device, such as is used for insufflation of the known agent sodium cromoglycate.

Compositions for administration by inhalation may be in the form of a conventional pressurised aerosol arranged to dispense the active ingredient either as an aerosol containing finely divided solid or liquid droplets. Conventional aerosol propellants such as volatile fluorinated hydrocarbons or hydrocarbons may be used and the aerosol device is conveniently arranged to dispense a metered quantity of active ingredient.

For further information on Formulation the reader is referred to Chapter 25.2 in Volume 5 of Comprehensive Medicinal Chemistry (Corwin Hansch; Chairman of Editorial Board), Pergamon Press 1990.

The amount of active ingredient that is combined with one or more excipients to produce a single dosage form will necessarily vary depending upon the host treated and the particular route of administration. For example, a formulation intended for oral administration to humans will generally contain, for example, from 0.5 mg to 2 g of active agent compounded with an appropriate and convenient amount of excipients which may vary from about 5 to about 98 percent by weight of the total composition. Dosage unit forms will generally contain about 1 mg to about 500 mg of an active ingredient. For further information on Routes of Administration and Dosage Regimes the reader is referred to Chapter 25.3 in Volume 5 of Comprehensive Medicinal Chemistry (Corwin Hansch; Chairman of Editorial Board), Pergamon Press 1990.

-37-

The size of the dose for therapeutic or prophylactic purposes of a compound will naturally vary according to the nature and severity of the conditions, the age and sex of the animal or patient and the route of administration, according to well known principles of medicine.

5 In using a compound for therapeutic or prophylactic purposes it will generally be administered so that a daily dose in the range, for example, 0.5 mg to 75 mg per kg body weight is received, given if required in divided doses. In general lower doses will be administered when a parenteral route is employed. Thus, for example, for intravenous administration, a dose in the range, for example, 0.5 mg to 30 mg per kg body weight will
10 generally be used. Similarly, for administration by inhalation, a dose in the range, for example, 0.5 mg to 25 mg per kg body weight will be used. Oral administration is however preferred.

It will be obvious to those skilled in the art to which the invention pertains, that various
15 changes and modifications may be made without departing from the scope of the invention defined by the claims.

P
R
V
0
3
-
0
7
-
2
2

Example 1

530 affected sib-pairs (ASPs) from 407 families with both sibs having CAD before 65 years were collected from hospitals in Sweden, Italy and Germany. All ASPs were recruited either from hospital discharge databases, existing research-based patient databases or from coronary
5 care units.

Probands were individuals with documented myocardial infarction (MI) or other symptomatic acute coronary syndrome (ACS; defined as hospitalisation for one of the following indications: a) unstable angina; b) thrombolysis for suspected MI (localised ST-elevation in 2 or more ECG leads); c) emergency revascularisation for control of typical ischaemic chest
10 pain at rest) first diagnosed before 65 years and confirmed by the physician. Affected sibs were individuals with confirmed MI, other symptomatic ACS as for the proband but in addition, chronic stable angina first diagnosed before 65 years and confirmed by physician, was included.

Preparation of genomic DNA from blood cells

15 Blood samples were stored at -80°C. Before extraction the samples were thawed at 37°C in a water bath. To extract the genomic DNA, using the PUREGENE DNA Isolation kit from Gentra Systems, the blood cells were added to 30ml of RBC lysis solution in a 50ml centrifuge tube. The mixture was inverted twice while incubating for 10minutes (min) at room temperature (RT). To separate the white cells from other lysed material, the mixture
20 was centrifuged in a RT-7™ from Sorvall Ltd. at 2,000X g for 10min. Most of the supernatant was removed leaving approximately 100ml of residual solution to resuspend the cells in by vigorously vortexing. Following resuspension 10ml of cell lysis solution were added and mixed by pipetting. To digest any RNA, 50ml of RNase A solution were added to the lysate, which was then incubated at 37°C for 15min. After RNA digestion, 3.3ml of protein
25 precipitation solution was added and the mixture was vortexed for 20seconds (s). To separate the proteins from the DNA, the mixture was again centrifuged at 2,000X g for 10min. The supernatant retaining the DNA was then poured off into another 50ml centrifuge tube containing 10ml of 100% isopropanol and mixed by inverting 50X. To separate the DNA from the aqueous solution, it was removed with an inoculating loop and washed in 10ml 70%

-39-

ethanol in a 50ml centrifuge tube. to remove any residual salt. The DNA was removed from the 70% ethanol using an inoculating loop and air dried briefly. To hydrate, the DNA was added to 1ml of DNA hydration solution in a 14ml centrifuge tube and this was rocked at RT overnight allowing the DNA to equilibrate. The resulting DNA samples were transferred to 5 1.5ml tubes and stored at -80°C. The concentration of extracted genomic DNA was quantified using the Fluorocount™ from Packard Instruments Co. The DNA was diluted to 4 ng/ml into a working plate.

Dispensing DNA from a working plate into several 96-well plates for subsequent polymerase 10 chain reaction (PCR) amplification reactions was performed using the Hydra™ 96 from Robbins Scientific, Inc. The Hydra™ was programmed to dispense 5µl of DNA from each well of a 'working plate' into the corresponding wells of the required number of PCR plates. Before dispensing, the Hydra™ was washed with water. Following dispensing the Hydra™ was sterilised by washing with water, with 2% chlorox industrial, and again with water. The 15 resulting PCR plates, containing 20ng of DNA in each well, were dried at 80°C for 1hr. The plates were stored at -20°C.

Microsatellite genotyping for genome scan

A total of 384 microsatellite markers were genotyped across the CAD ASP families. The majority of these were from the linkage mapping set 2.0 (Applied Biosystems). Additional 20 markers were included to replace those excluded by error checking procedures. The amplification of microsatellite markers by PCR and subsequent pooling of the amplified products into panels were carried out using a RapidGene™ automated system from Oxagen Ltd. The PCR plates contained 20ng of dried genomic DNA in each well. The 1.5ml tubes contained 5mM of appropriate 'forward' oligonucleotide, 5mM of the corresponding 'reverse' 25 oligonucleotide, 1X PCR buffer II, 2.5mM MgCl₂, 1mM dNTP, and 0.06u/ml AmpliTaq Gold™ DNA polymerase in final volume of 1.2ml. During the run, 10µl of the appropriate PCR reaction mixture were dispensed into the wells of the plates. Following amplification the reactions were pooled into panels and the pool plates were sealed and stored at 4°C. Pooled DNA fragments generated from the amplification of microsatellite markers were separated by 30 capillary electrophoresis using an ABI 3700 automated sequencer (PE Applied Biosystems,

Inc.). Products were sized using the programme ABI GENESCAN (version 3.0) (PE Biosystems) and genotypes assigned semi-automatically using the GENOTYPER software (version 2.0).

5 *Linkage Analysis Chromosome 3*

In a genome wide screen with 384 markers (average marker distance 9.2 cm) 530 affected sib-pairs were analysed from 407 families. In addition marker genotypes for at least one parent or unaffected sibs were available in 110 families.

- 10 The genotype data for chromosome 3 include 23 markers (see Fig 1) with an average distance of 9.8 cM. Linkage analysis was performed in Genehunter-Plus version 1.3 (Kruglyak et al, Am. J. Hum. Genet. 58, 1347-1363 (1996) and Kruglyak and Lander. Journal of Computational Biology 5:1-7 (1998)) using the 'all' scoring function, followed by calculation of LOD-scores in KAC as described by Kong and Cox Am. J. Hum. Genet. 61, 1179-1188
- 15 (1997). A maximum LOD-score of 3.45 was obtained at 9 cM, with the position given as the distance from the most telomeric marker D3S1297 (Fig 1). In table 2, LOD-scores for the three markers overlapping the peak are displayed.

Position	LOD	Marker
0.0	2.51	D3S1297
9.0	3.45	-
14.1	3.21	D3S1304
27.8	0.45	D3S1263

- 20 To assess the statistical significance of the obtained LOD-score we simulated marker genotype data under a model with no genetic effect, preserving the family structures and missing data patterns of the families in the study, using the observed marker allele frequencies. In 3600 replicates no LOD-scores as large as the one observed for the real data was observed for chromosome 3, indicating a p-value less than 0.001. Consideration of the
- 25 LOD-scores obtained on all autosomal chromosomes in each replicate resulted in an empirical genome-wide p-value of 0.01.

Genetic marker distances were obtained from the Marshfield map and translated to recombination fractions using the Kosambi mapping function. The marker allele frequencies were estimated from all genotyped individuals in the study.

- 5 Prior to the linkage analysis the genotype data went through quality control. This includes identification of half-sibs or unrelated individuals among the putative sib pairs using RELPAIR (Boehnke *et al.*, Am. J. Hum. Genet. 61:423-429. 1997), detection of Mendelian inconsistencies with PEDCHECK (O'Connell *et al.*, Am. J. Hum. Genet. 63:259-266 1998) and retyping of inconsistent marker-family combinations. Detected half-sib pairs were
10 excluded from the linkage analysis described here.

Associated gene identification

The highest linkage was found between makers D3S1297 and D3S1304, within this region lies the BHLHB2 gene. Further SNP mapping identified this gene as being associated with the
15 CAD.

Example 2

Identification of polymorphisms in BHLHB2, a coronary artery disease (CAD) susceptibility gene on chromosome 3p

20

According to the Ensembl genome assembly (build 31), the BHLHB2 gene consists of 5 exons covering a genomic distance of 5.7 kb. BLAST analysis using BHLHB2 cDNA suggests that there might be one or two additional 5' untranslated exons. The gene was screened for polymorphisms, and a selection of the identified polymorphisms were
25 subsequently tested for association to CAD in a TDT material.

1. DNA sequencing of BHLHB2 exons 1-5

Individual BHLHB2 exons were PCR amplified from genomic DNA of 24 unrelated individuals, using oligonucleotide primers designed in flanking intronic sequences.

The PCR amplified products were then sequenced and SNPs identified by Dye-primer sequencing as described in the ABI protocol P/N 402114 using ABI 3700 automated sequencers. Genotyping of informative SNPs was performed by real-time PCR using the TAQMAN™ technology from PE Biosystems.

5 The SNPs (or other polymorphisms) and their frequencies are shown in Table 3. Sequence flanking the polymorphisms is shown in Table 4, Table 5 shows sequence adjacent the polymorphisms.

2. Mining public SNP databases

¹⁰ Additional public domain SNPs in the BHLHB2 gene were identified (dbSNP; <http://www.ncbi.nlm.nih.gov/SNP/>).

SNP genotyping

Five SNPs discovered in Step 1 (rs1110261, rs2271566, rs2671757, rs908078, and DEC1az1494), were genotyped in at least 92 UK blood donor control DNAs obtained for validation of SNPs in the TDT collection. The results are shown in Table 3.

Microsatellite genotyping

One marker discovered in Step 1, DEC1azGTrep, was genotyped in a number of individuals from the TDT collection. The results are shown in Table 3.

Table 3

Internal identifier	Identifier	Position in gene (Genbank ID: AB043885)	*Allele change	Allele frequency (# of individuals)	"Single" p- value in fist TDT analysis
1	rs1110261	2176bp	G/A	no info	0.7362
2	rs1104976	2388bp	A/C	unconfirmed	
3	rs2271566	3643bp	C/T	C=84% / T=16% (3300)	0.9943
4	az0001116	3944bp	C/T/-	not polymorphic	
5	rs2671757	5112bp	C/T	C=81% / T=19% (3304)	0.0087
6	DEC1azCArep	5112-5133bp	(CA)n	no info	
7	rs908078	7002bp	T/C	T=84% / C=16% (3268)	0.7346
8	rs3205438	7082bp	A/G	not polymorphic	
9	DEC1azGTrep	7746-7775bp	(GT)n	no info	0.3538
10	DEC1az1131	7880bp	G/C	C=83% / T=17% (48)	
11	DEC1az1136	7885bp	G/T	G=67% / T=32% (48)	
12	DEC1az1140	7889bp	G/A	G=83% / A=17% (48)	
13	DEC1az1494	8243bp	A/G	A=81% / G=19% (48)	0.0660
14	rs1801739	8677bp	A/T	not polymorphic	
15	rs1058377	8905bp	A/C	not polymorphic	
16	rs1058380	8943bp	G/A	not polymorphic	
17	rs1058395	9065bp	A/T	not polymorphic	

TDTLIKE Terwilliger, (Am J Hum Genet 56:777-787, 1995).

Table 4

Internal identifier	5'flanking sequence
1	CCGCGAACAGCGCCGCCGAACCTCCCAACAGGCAGGGAGAGGGCCGATCCGGGCTGGCAC
2	GGAGGCGACCGGGGCGCCTCTGCTCCGGAGGGGGCAGAGGAGGGAGGGAGCGCCGGGCAC
3	CCTCATCCAGACGCTCCGCTAGTGCAGACAGGAGCGCGCAGTGGCCCCGGCTCGCCGCGC
4	CAGGATGTACCCTGCCACATGTACCAAGTGTACAAGTCAAGACGGGGAATAAAGCGGAG
5	AGACAGGTGGCACCCTTTTTTTAAATTATTTAATTATATATATATATTATATATATATA
6	AGACAGGTGGCACCCTTTTTTTAAATTATTTAATTATATATATATATTATATATATATA
7	ATCAGACCCAGCTCCCAAAGTGATGGACTTCAAGGAAAAACCCAGCTCTCCGGCCAAAGG
8	ACTGCGTGCCAGTCATCCAGCGGACTTTCGCTCACTCGAGTGGGGAGCAGAGCGGCAGCG
9	CTGCAAGATTGTTGCATTGTGTATACTGAGATAATCTGAGGCATGGAGAGCAGATTCAGG
10	CTTTAAAGCTCCTTTTGGCATAGGGAAGTCACGAAGGATTGCTTGACATCAGGAGACTTG
11	AAGCTCCTTTTGGCATAGGGAAGTCACGAAGGATTGCTTGACATCAGGAGACTTGGGGGG
12	TCCTTTTGGCATAGGGAAGTCACGAAGGATTGCTTGACATCAGGAGACTTGGGGGGGATT
13	CATAGCACGGTAGTGGTTTGGGGAGGTTCCGCAGGTCTGCTCCCCACCCCTGCCTCGGA
14	CAACGGCATATGGAGTGTCTTATTGCTAAAAAGGATTCCGTCTCCTTCAAAGAAGTTTT
15	TATATAAATATATTA AAAAGGAAAATGTTTCAGATGTTTATTTGTATAATTACTTGATTC
16	TATTTGTATAATTACTTGATTCACACAGTGAGAAAAATGAATGTATTCCTGTTTTTGAA
17	GAAGGTGTAAATTGTAAATATTTTATCTATGAGTAAATGTTAAGTAGTTGTTTTAAAA

Table 5

Internal identifier	3'flanking sequence
1	CCACGTCCCCGGGTCTCAGTCCCTGGGTCAACGCAGAGAGGCTCGCGGTCCGCGGTGCT
2	GCGCCCGGTATCTTTCTGCGCTTGACTGGCCGGGAGGAAGGGGGTGACACTGGGGCACCC
3	ATGGAGCGGATCCCCAGCGCGCAACCACCCCCCGCCTGCCTGCCAAAGCACCGGGACTG
4	GAGGACAGCAAGGTAAGCAAGTGCACCCCTAGGGACCCTGCGCTCAGCCCCCTCGCGCGCG
5	ACACACACACACACACACATATAGAGTGTATCTGTCTATCTTTTTAAAGCATGACC
6	TATATGAGTGTATCTGTCTATCTTTTTAAAGCATGACCTACAGACTTAAACAGCCAAC
7	TCGGAAGGTCTGGGAAAACTGCGTGCCAGTCATCCAGCGGACTTTCGCTCACTCGAGT
8	CACGGACACAGACAGTGGCTATGGAGGAGAATCGGAGAAGGGCGACTTGCGCAGTGAGCA
9	ATGTGCGTGTGCGTGACATGTGTGCCTGCGTGTTGGTATAGGACTTTAAAGCTCCTTTT
10	GGGGGATTGTAGCAGACGTCTGGGCTTTTCCCCACCCAGAGAATAGCCCCCTTCGATACA
11	ATTGTAGCAGACGTCTGGGCTTTTCCCCACCCAGAGAATAGCCCCCTTCGATACACATCA
12	TAGCAGACGTCTGGGCTTTTCCCCACCCAGAGAATAGCCCCCTTCGATACACATCAGCTG
13	GAATAAAGAGAATGTAGTCCCTACTCAGGCTTTCGTAGTGATTAGCTTACTAAGGAACT
14	TTTTTGGTCCAGAGTACTTGTTTTCCCGATGTGTCCAGCCAGCTCCGCAGCAGCTTTTCA
15	CACAGTGAGAAAAATGAATGTATTCCTGTTTTGAAGAGAAGAATAATTTTTTTTTTCT
16	AGAAGAATAATTTTTTTTTCTCTAGGGAGAGGTACAGTGTTTATATTTTGGAGCCTTCC
17	ACTTAATAAAATAATTCTTTTCTGTGGAAGAGAAAGATGGTCTCCTGAGTTTTTAAAA

CLAIMS:

1. A method of identifying a test compound that modulates the expression of BHLHB2 gene comprising:

contacting a cell capable of expressing BHLHB2 gene with a test compound; and

5 determining the level of expression of the BHLHB2 gene in the presence of the test compound, wherein a decrease or an increase in expression of the BHLHB2 gene, as compared to the level of expression of a BHLHB2 gene in the absence of the compound, is indicative that the test compound modulates BHLHB2 gene expression.

10 2. A method of identifying a test compound that modulates the activity of a BHLHB2 protein comprising:

contacting the BHLHB2 protein with a test compound; and

determining the level of activity of the BHLHB2 protein in the presence of the compound, wherein a decrease or an increase in BHLHB2 protein activity, as compared to the
15 level of activity of the BHLHB2 protein in the absence of the compound, is indicative that the test compound modulates BHLHB2 protein activity.

3. A method of treating a subject having CAD comprising administering an effective amount of the compound identified in claims 1 or 2.

20

4. Use of a compound identified in claims 1 or 2 in the preparation of a medicament for the treatment of CAD.

5. A method of treating a patient suffering from CAD comprising administering to said
25 patient an effective amount of an antibody specific for protein BHLHB2.

6. A method of treating a patient suffering from CAD comprising administering to the patient an effective amount of an antisense molecule capable of binding to the mRNA of gene BHLHB2, wherein binding of the antisense molecule to the mRNA causes a decrease in
30 expression of the protein product of gene BHLHB2.

-47-

7. A pharmaceutical composition comprising the compounds identified in claims 1 or 2, and a pharmaceutically acceptable adjuvant, diluent or carrier.

8. A method of making a pharmaceutical composition comprising:

- 5 contacting a cell capable of expressing BHLBH2 with a test compound;
 determining the level of expression of BHLBH2 in the presence of the test
compound; wherein a decrease or an increase in expression of BHLBH2, as compared to the
level of expression of BHLBH2 in the absence of the compound, is indicative that the test
compound modulates BHLBH2 gene expression; and
10 formulating the test compound that modulates BHLBH2 into a pharmaceutical
composition.

9. A method of making a pharmaceutical composition comprising:

- contacting a BHLBH2 protein with a test compound;
15 determining the level of activity of the BHLBH2 protein in the presence of the
compound, wherein a decrease or an increase in BHLBH2 protein activity, as compared to the
level of activity of the BHLBH2 protein in the absence of the compound, is indicative that the
test compound modulates BHLBH2 protein activity; and
 formulating the test compound that modulates BHLBH2 protein activity into a
20 pharmaceutical composition.

10. A method for determining if a BHLBH2 gene has an altered level of gene expression comprising:

- comparing the level of BHLBH2 gene expression in a cell from a patient having CAD
25 with a control cell; and
 determining the level of expression of the BHLBH2 gene in both cells, wherein a
decrease or an increase in expression of the BHLBH2 gene, as compared to the level of
expression of the BHLBH2 gene in the control cell, indicates that the BHLBH2 has altered
gene expression.

30

11. A method for determining the level of a BHLBH2 protein in a CAD patient compared to a control comprising:

-48-

comparing the protein level of BHLBH2 in a cell from a patient having CAD with a control cell; and
determining the level of the BHLBH2 protein in both cells.

- 5 12. A method of identifying a binding partner of the BHLHB2 protein comprising:
contacting a BHLHB2 protein with a test target protein; and
determining if the test target protein can interact with the BHLHB2 protein, wherein
interaction of the test target protein with BHLBH2 indicates that the test target protein is a
BHLBH2 binding partner.
- 10 13. The method of claim 12, wherein the method further comprises:
contacting a gene encoding the test target protein with a test compound; and
determining the level of expression of the test target gene in the presence of the test
compound, wherein a decrease or an increase in test target gene expression, as compared to
15 the level of expression of the test target gene in the absence of the compound, is indicative
that the test compound modulates expression of the test target gene and is useful in the
treatment of CAD.
14. The method of claim 12, wherein the method further comprises:
20 contacting the test target protein with a test compound; and
determining the level of activity of the test target protein in the presence of the test
compound, wherein a decrease or an increase in test target protein activity, as compared to the
level of activity of the test target protein in the absence of the compound, is indicative that the
test compound modulates test target protein activity and is useful in the treatment of CAD.
- 25 15. The method of any of claims 13 or 14, wherein the test compound is formulated into a
pharmaceutical composition.
16. A method of treating a subject having CAD comprising administering an effective
30 amount of the compound identified in claims 13 or 14.

17. Use of a compound identified in of any of claims 13 or 14 in the preparation of a medicament for the treatment of CAD.

18. A method of identifying other components of the CAD biochemical pathway of which
5 BHLHB2 is a component.

19. A method of diagnosing CAD, or a susceptibility thereto in a subject, the method comprising:

determining the level of mRNA of BHLHB2 in a sample from a subject; and
10 comparing the level of mRNA of BHLHB2 in the sample with a control, wherein a decrease or an increase in the level of mRNA of BHLHB2 in the sample compared to the control indicates that the subject has CAD , or a susceptibility thereto.

20. A method of diagnosing CAD or a susceptibility thereto in a subject, the method
15 comprising:

determining the level of a BHLHB2 protein in a sample from a subject; and
comparing the level of BHLHB2 protein in the sample with a control, wherein a decrease or an increase in the level of the protein in the sample compared to the control indicates that the subject has CAD , or a susceptibility thereto.

-50-

Abstract

The present invention describes a Coronary artery disease (CAD) susceptibility gene and the use of this gene in the development of novel therapies to treat CAD.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

Sequence listing:

SEQ ID No:1

CCGCGAACAGCGCCGCCGAACCTCCCAACAGGCAGGGAGAGGGCCGATCCGGGCTGGCACR
5 CCACGTCCCCGGGGTCTCAGTCCCTGGGTCAACGCAGAGAGGCTCGCGGTCCGCGGTGCT

SEQ ID No:2

GGAGGCGACCGGGGCGCCTCTGCTCCGGAGGGGGCAGAGGAGGGAGGGAGCGCCGGGCACM
GCGCCCGGTATCTTTCTGCGCTTGACTGGCCGGGAGGAAGGGGGTGACACTGGGGCACCC
10

SEQ ID No:3

CCTCATCCAGACGCTCCGCTAGTGCAGACAGGAGCGCGCAGTGGCCCCGGCTCGCCGCGCYATGGAGCGG
ATCCCCAGCGCGCAACCACCCCCCGCCTGCCTGCCCAAAGCACCGGGACTG

15 SEQ ID No:4

CAGGATGTACCCTGCCACATGTACCAAGTGTACAAGTCAAGACGGGGAATAAAGCGGAGN
GAGGACAGCAAGGTAAGCAAGTGCACCCCTAGGGACCCTGCGCTCAGCCCCTCGCGCGCG

SEQ ID No:5

20 AGACAGGTGGCACCCGTTTTTTAAATTATTTAATTATATATATATATTATATATATATAY
ACACACACACACACACACATATATGAGTGTATCTGTCTATCTTTTTAAAAGCATGACC

SEQ ID No:6

AGACAGGTGGCACCCGTTTTTTAAATTATTTAATTATATATATATATTATATATATATA(CA)_n
25 TATATGAGTGTATCTGTCTATCTTTTTAAAAGCATGACCTACAGACTTAAACAGCCAAC

SEQ ID No:7

ATCAGACCCAGCTCCCAAAGTGATGGACTTCAAGGAAAAACCCAGCTCTCCGGCCAAAGGY
TCGGAAGGTCCTGGGAAAACTGCGTGCCAGTCATCCAGCGGACTTTCGCTCACTCGAGT
30

-52-

SEQ ID No:8

ACTGCGTGCCAGTCATCCAGCGGACTTTTCGCTCACTCGAGTGGGGAGCAGAGCGGCAGCGR
CACGGACACAGACAGTGGCTATGGAGGAGAATCGGAGAAGGGCGACTTGCGCAGTGAGCA

5 SEQ ID No:9

CTGCAAGATTGTTGCATTGTGTATACTGAGATAATCTGAGGCATGGAGAGCAGATTCAGG(GT)_n
ATGTGCGTGTGCGTGACATGTGTGCCTGCGTGTGGTATAGGACTTTAAAGCTCCTTTT

SEQ ID No:10

10 CTTTAAAGCTCCTTTTGGCATAGGGAAGTCACGAAGGATTGCTTGACATCAGGAGACTTGS
GGGGGATTGTAGCAGACGTCTGGGCTTTTCCCCACCCAGAGAATAGCCCCCTTCGATACA

SEQ ID No:11

AAGCTCCTTTTGGCATAGGGAAGTCACGAAGGATTGCTTGACATCAGGAGACTTGGGGGGK
15 ATTGTAGCAGACGTCTGGGCTTTTCCCCACCCAGAGAATAGCCCCCTTCGATACACATCA

SEQ ID No:12

TCCTTTTGGCATAGGGAAGTCACGAAGGATTGCTTGACATCAGGAGACTTGGGGGGGATTR
TAGCAGACGTCTGGGCTTTTCCCCACCCAGAGAATAGCCCCCTTCGATACACATCAGCTG
20

SEQ ID No:13

CATAGCACGGTAGTGGTTTGGGGAGGTTTCCGCAGGTCTGCTCCCCACCCCTGCCTCGGAR
GAATAAAGAGAATGTAGTTCCCTACTCAGGCTTTCGTAGTGATTAGCTTACTAAGGAACT

25 SEQ ID No:14

CAACGGCATATGGAGTGTCTTATTGCTAAAAAGGATTCCGTCTCCTTCAAAGAAGTTTTW
TTTTTGGTCCAGAGTACTTGTTTTCCCGATGTGTCCAGCCAGCTCCGCAGCAGCTTTTCA

SEQ ID No:15

TATATAAATATATTAAGGAAAATGTTTCAGATGTTTATTGTATAATTACTTGATTCTM
CACAGTGAGAAAAAATGAATGTATTCTGTTTTGAAGAGAAGAATAATTTTTTTTTTCT

5 SEQ ID No:16

TATTTGTATAATTACTTGATTACACAGTGAGAAAAAATGAATGTATTCTGTTTTGAARAGAAGAATAATTTT
TTTTTCTCTAGGGAGAGGTACAGTGTTTATATTTGGAGCCTTCC

SEQ ID No:17

10 GAAGGTGTAATAATTGTAATATTTTTATCTATGAGTAAATGTTAAGTAGTTGTTTTAAAW
ACTTAATAAAATAATTCTTTTCTGTGGAAGAGAAAGATGGTCTCCTGAGTTTTTTAAAA

SEQ ID NO: 18

MERIPSAQPPACLPKAPGLEHGDLPGMYPAHMYQVYKSRRGIKRSSEDSKETYLPH
15 RLIEKKRRDRINECIAQLKDLLPEHLKLTTLGHLEKAVVLELTLKHVKALTNLIDQQQ
QKIALQSGLQAGELSGRNVETGQEMFCSGFQTCAREVLQYLAKHENTRDLKSSQLV
THLHRVVSELLQGGTSRKPSDPAPKVMDFKEKPSSPAKGSEGPKNVFPVIQRTFAHS
SGEQSGSDTDTDSGYGGESEKGDRLSEQPCFKSDHGRRFTMGERIGAIKQSEEPPTK
KNRMQLSDDEGHFTSSDLISSPFLGPHPHQPPFCLPFYLIPPSATAYLPMLEKCWYPTS
20 VPVLYPGLNASAAALSSFMNPDKISAPLLMPQRLPSPLPAHPSVDSSVLLQALKPIPL
NLETKD

SEQ ID NO: 19

1 ggacaccggg ccatgcacgc ccccaactga agctgcatct caaagccgaa gattccagca
25 61 gcccagggga ttcaaagag ctcagactca gaggaacatc tgcggagaga cccccaagc
121 cctctccagg gcagtcctca tccagacgct ccgctagtgc agacaggagc gcgcagtggc
181 cccggctcgc cgcgccatgg agcggatccc cagcgcgcaa ccacccccg cctgcctgcc
241 caaagcaccg ggactggagc acggagacct accagggatg taccctgccc acatgtacca
301 agtgatacag tcaagacggg gaataaagcg gagcaggagc agcaaggaga cctacaatt
361 gccgcaccgg ctcacgaga aaaagagacg tgaccggatt aacgagtgc tgcaccagct
421 gaaggatcct ctaccgaac atctcaact tacaacttg ggtcacttg aaaaagcagt
481 ggttcttgaa ctaccttga agcatgtgaa agcactaaca aacctaatg atcagcagca
541 gcagaaaatc attgccctgc agagtgggtt acaagctggt gagctgtcag ggagaaatgt
601 cgaaacaggt caagagatgt tctgctcagg ttccagaca tgtgccggg aggtgcttca
35 661 gtatctggcc aagcagaga acactcggga cctgaagtct tcgcagctg tcaccacat
721 ccaccgggtg gtctcggagc tgctgcaggg tggtaacctc aggaagccat cagaccagc
781 tccaaagt atggactca aggaaaaacc cagctctcc gccaaaggt cggaaggtcc
841 tgggaaaaac tgcgtgccag tcatccagcg gacttctcct cactcgagt gggagcagag
901 cggcagcgac acggacacag acagtggcta tggaggagaa tcggagaagg gcgacttgcg

-54-

961 cagtgcagcag ccgtgcttca aaagtgacca cggacgcagg ttacgatgg gagaaaggat
 1021 cggcgcaatt aagcaagagt ccgaagaacc cccacaaaa aagaaccgga tgcagcttcc
 1081 ggatgatgaa ggccatttca ctacgagtga cctgatcagc tccccgttcc tgggcccaca
 1141 cccacaccag cctcctttct gctgcccctt ctacctgac ccaccttcag cgactgccta
 5 1201 cctgccccatg ctggagaagt gctggatcc caccctcagt ccagtgcctat acccaggcct
 1261 caacgcctct gccgcagccc tctctagctt catgaacca gacaagatct cggctcccct
 1321 gctcatgccc cagagactcc ctctcccctt gccagctcat ccgtccgtcg actcttctgt
 1381 ctgctccaa gctctgaagc caatcccccc tttaactta gaaaccaaag actaaactct
 1441 ctaggggatc ctgctgcttt gctttccttc ctgctactt ctaaaaaagc aacaaaaaag
 10 1501 tttttgtgaa tgctgcaaga ttgtgcatt gtgtatactg agataatctg aggcatggag
 1561 agcagattca ggggtgtgtgt gtgtgtgtgt gtgtgtgtgt gtatgtgcgt gtgcgtgcac
 1621 atgtgtgcct gcgtgttgggt ataggacttt aaagctcctt ttggcatagg gaagtcacga
 1681 aggattgctt gacatcagga gacttggggg ggattgtagc agacgtctgg gcttttcccc
 1741 acccagagaa tagccccctt cgatacacat cagctggatt tcaaaagct tcaaagtctt
 15 1801 ggtctgtgag tcactcttca gtttgggagc tgggtctgtg gctttgatca gaaggctact
 1861 tcaaaagagg gctttccagg gctcagctcc caaccagctg ttaggacccc acccttttgc
 1921 ctttattgtc gacgtgactc accagacgtc ggggagagag agcagtcaga ccgagcttcc
 1981 tgctaacatg gggaggtagc aggcactggc atagcacggt agtggtttgg ggaggtttcc
 2041 gcaggtctgc tccccacccc tgcctcggaa gaataaagag aatgtagttc cctactcagg
 20 2101 ctttctagtg gattagctta ctaaggaact gaaaatgggc ccttgtaca agctgagctg
 2161 ccccgaggag agggaggagt tccttgggct tctggcacct gtttctaggc ctaaccatta
 2221 gtacttactg tgcagggaac caaccaagg tctgagaaat gcggacaccc cgagcgagca
 2281 ccccaaagtg cacaagctg agtaaaaagc tgcccccttc aaacagaact agactcagtt
 2341 tcaattcca tctaaaaact cctttaacc aagcttagct tctcaaaggc ctaaccaagc
 25 2401 ctggcacccg ccagatcctt tctgtaggct aattctctt gcccaacggc atatggagtg
 2461 tcttattgc taaaaaggat tccgtctcct tcaaagaagt ttatttttg gtccagagta
 2521 ctgttttcc cgatgtgtcc agccagctcc gcagcagctt tcaagatgc actatgctg
 2581 attgtgatc gtgtttaac ttttcttt cctgtttta ttttggtatt aagtcgttgc
 2641 ctttattgt aaagctgtta taaatatata ttatataat atattaaaa ggaaaatgtt
 30 2701 tcagatgatt atttgtataa ttacttgatt cacacagtga gaaaaatga atgtatctt
 2761 gtttttgag agaagaataa tttttttc tctagggaga ggtacagtgt ttatatttg
 2821 gagccttctt gaagggtgaa aattgtaat attttatct atgagtaaat gtaagtagt
 2881 tgttttaaaa tacttaataa aataattctt ttcctgtgga ag

35 SEQ ID NO: 20

1 tagatagtaa cagtatagac tcattcagca cacagtgtaa ggccaccaag atgtgagtc
 61 ccaataggaa agaagagaaa gatcccatct ttctcttga cgaaattatg ttctctcccc
 121 acttagaaga gacaaaacag cagggacggc tacaaaatgg caggattacc cagtgcagct
 181 gctcagtgcc acattgcaca actccagagg gtgctctcac acagactgca atgtgaatag
 40 241 ctccccctgg agttgagcgg tgcacaaccc aaacaaccct accagggaac cgtaagtata
 301 gtgattaaga gcacaagctt tggagtcaa caaacctggg gccctgtctc agactgaatc
 361 ccagactata taacctgggg caggttattt agcctccttg agcctcaatt ttcaaacag
 421 aatatgttat acatccctag ggcctatttc agtctctggc attgaataac aataaacacg
 481 tgtcaaatgc atcagagctaa tactagtacc tgcctaaaag gtggtggtga ggagcaaga
 541 aattgacata cgaaagggtt ttaatatcta caaaagatga cagactgaaa tagtaacatt
 45 601 ctccaggatg gaaagatcta tgccacgtag gaattgttg ttcaaattat tattttttaa

661 aagccataag ctatggagag aataacagag tttgtttg tttttaac ctgatacgg
721 ggaagtctgg taggatggga accgttctg aagcttgaga atacattcgg aacaaataa
781 aagtaggaaa tgcatacatt ttgtattta cagttgtagg gcaggttaagg gcaaacgta
841 ttgataaact caaagaattt taagaatgat attgtcatga gccatgagcc atggaagctg
5 901 caaggtttgg gctccctcct gccatcgggt ggggggtgtc ggcggaagg ggtacgtg
961 ctgtcctc ccaagtgtta ttctctgctg gaaccgtggt ttgacgcag ccgtgacagc
1021 ccttcacct tattaatac acacaatgaa gcaggctgcc caagattgaa agcgcgttc
1081 cagggcgag cccggtcccc gccttggtcc agcaaaagta cattctggca gggagggtac
1141 tctgagcccc gaagttaggt catgccagtt agcagttaga aaaggaggca ttctcacac
1201 ttgctaaaga gttgacgct tgtgaatat aaacaggaag cgggagctct ccaaggcgac
1261 ctgaaggaaa tacactgcca gtttggtcaa ctgtgaatg ccagttctct aggtcgtcaa
1321 gggcactgcc agttaagact gatccccctaa ctgcaccga ggctactact ctgcgccag
1381 tctcggcagt gacccattt ttttcaat cctcccaca gccaggtcac tcagcagtg
1441 ggtgactgac ccgaagcga gtgggaagg cttacagtc tcttcaact cccgaact
15 1501 gcagggcagg ggagcgcaga ggtcagtga atcccagcc aggcctatt ttagtcag
1561 gtttccactg gctccctcct ctgactgtat cagcgtgct caccgcgg gattggagt
1621 tcagctgtt tcctgtcc cggctgagt agaaggcac agagcccgga ctttccggg
1681 ggaggtgggt ggggcccccc ggcagcctaa gtgagatgc agagatctgg ggccgctgga
1741 ggaggtcct cggccccggg ctgggggtt tgggttagt tccccccgc ttctacct
20 1801 aggcactcg gttacaatga aattaaaaa aaaacacaaa gcaacacaag ttctattgt
1861 tttcccctc tgcagccag tggcctgat acaatttcc cagggtgcc ttctttat
1921 tgcataatt aacctctcc tagaggttc gcaagtcaa gggcaggatc cgtgtcgat
1981 tcatcaacta gtgcagcgc tagcgcagag cagggggctc aagaaatag tgcgaccgc
2041 atgattgcta agatttccct gtaacaggcg gttttttt ctgtctctc tcatccctc
25 2101 tgccctgggt gcagaccgc aacagcgcg ccgaacttc caacaggcag ggagaggggc
2161 gatccgggt ggacgccac gtccccggg tctcagtc tgggtcaac cagagaggct
2221 cgcggtccg ggtgtacga cctgagccc gccacgtga gcgtcacgc ctgcgtgct
2281 gcagggcagg cgcgcgcgc gcggtgtg aacgtcag ccggagggga ggcgaccggg
2341 gcgcctctgc tccggagggg gcagaggagg gagggagcgc cgggcacagc gcccggtac
30 2401 ttctgctgct tgactggccg ggaggaagg ggtgacact gggcaccag ggggtgcgc
2461 aaccgggtgc ccgccccc gcaggtgtg cccaggagc gagctcctg gagcgaggg
2521 cctcggctc acacctcca cgtcaggtg cgcgcggct gctcggcgc agcctcagct
2581 gcccctgcg ttggcgtc gcccgttc catgggtga catccgcc cccctcgt
2641 cctcccaa ggcgggcaat tctggagc gaggtgagc agtgggggca gggaagccag
35 2701 gacggaaaga aacccagcc tctggggaag gctggggggc cgggcgact cctccgagc
2761 gcgcccgtc ctcaagcgg gccgggggg ggaggaggaa gagggctgg ctggagctag
2821 caaggggata ttctctccc ggttgatc agacggggc ggtccgtc tccccgtc
2881 cctccagga gacgggaact tacttatt cctggggca ggttcgcca cgttaccac
2941 ttccccct ccccagcac cccgtccct tccagctc gcgccccca ccaactggg
40 3001 caggaccag gtcgtgtgc caccctct tgggggaaag gcggccgcag ccgcagacac
3061 ctggggggcc gggctggggg tgggggtcc ctgacagcc cggagcgtt gtccaacag
3121 tgagactcat gtgatgaag cgggggagg cgggcaggtc gctcctcc tcccggcag
3181 tggccagac gctctggagt cacaggtag aacacgtag tccaaccac cactcgtc
3241 ccattaacc cagccgcag ctctccatt actctcggc ttgcccccc accccccac
45 3301 tccgcctaa cggcccccc ctcaccgct cctccgcc tcccgcgg cccacttct
3361 cattcactg gctgcacgg cgcagacga ccgcgcagg agcacacac gccagtctg
3421 gcgctgagc ggagccagag gcccggggga caccgggcca tgcagcccc caactgaagc
3481 tgcactcaa agccgaagat tccagcagc caggggatt caaagagtc agactcag

3541 gaacatctgc ggagagaccc ccgaagccct ctccagggca gtctcatcc agacgtccg
 3601 ctagtgcaga caggagcgcg cagtggcccc ggctcgcgc gccatggagc ggatccccag
 3661 cgcgcaacca cccccgcct gcctgccccaa agcaccggga ctggagcacg gagacctacc
 3721 agggtaagt ggcactcctt ggcccttcaa gcctcaactg cagccccatg ttatgcgcca
 5 3781 ctctcaactt ggagcagctc cggcgccacc gggagggtct tgcgggcag aacgcctgca
 3841 gccgtgcgca agcagtcacg acccttctg gtctctctc ctgcaggatg taccctgcc
 3901 acatgtacca agtgataag tcaagacggg gaataaagcg gagcgaggac agcaaggtaa
 3961 gcaagtgcac ccctagggac cctgcgctca gccctcgcg cgcgctgagt ttccaagaaa
 4021 agttttctc cttgaggtt ggcgagggga tgcaggggtg gggctcggg cctctctga
 10 4081 gtgacttga aaagaaagg cagacgatgg tctgggtgc tactctgcta ctctgtcct
 4141 ctgccggact gttgtcgcg ggctgggagg ataggcttct catctcttc cccaagcgc
 4201 caccgcctcc cgtgcgtct tgcaggagac ctacaaattg ccgcaccggc tcatcgagaa
 4261 aaagagacgt gaccggatta acgagtgcac cgcacagctg aaggatctcc taccgaaca
 4321 tctcaaactt acagtaagt agaagctggc cccttccaa ccagtcctt tgcagagg
 15 4381 gggggcgggt cactcgcgc ctgcaggctc cgcggggaac tgcagaccag actggcggt
 4441 cagagctggc ggggtgctct gcggtgggt cctctccac agtctggcg tttctggct
 4501 gactcatgca aatgaggcga gaaccactg gacctctca agttggctcc aacaccaact
 4561 ggccttctt ttacagggt taactctgg tgatttcag gattccctag ttttctcac
 4621 ttaaaggaac acgccaagaa acacacacat ttatttacc cttccctc taccactccc
 20 4681 cccaccttt ttgtgtgt tgcataaa ggggacttgc ttgtgtgt gattttacg
 4741 gcctgtcct tctgacagt agccctcggc tactcgcag ctttgggtg gggaaatgga
 4801 aaaaaaaaa atagtattt gggaggaaca aaaaagcctg gatgagctgg aagttctgaa
 4861 aggggtgaag gaaagaagta gggaaagcct ctgggtgcc agacgtaata gaggtccctc
 4921 tctgagttga caccactgca ggaaaatgt atctctggat tcccctgagt taaggtcggc
 25 4981 agaggctgct ggagaaatt atctgtcaa ggatttctt gaggtagtg cagcttccaa
 5041 aatgcctcca aagacagggt gcaccggtt tttaaatta tttaattata tatatatatt
 5101 atatatatat atacacacac acacacacac acatatatga gtgtatctgt ctatctttt
 5161 aaaagcatga cctacagact taaaacagcc aaccaggaaa cactattcca cttttttt
 5221 tcttttca tttctattg ctaataatg tccaaagggt gggacttctc tgacctcacc
 30 5281 gctgactaag gctttttc ttccagactt tgggtcactt ggaaaaagca gtggttcttg
 5341 aacttacct gaagcatgtg aaagcactaa caaacctaat tgatcagcag cagcagaaaa
 5401 tcatgccct cgagagtgggt ttacaagctg gtgagtgtg attctggcta tgcctctt
 5461 aagagttga gtgcaaatag agagcccggt ggctcaacat ctgatgtaat aaacattact
 5521 gcaacaaatt gcttatgcac gtctattggg ggagatgcag tttatctt cctaaaagg
 35 5581 cagactttc attgaaatg cattgtgcca cctggcatga atacgtatta gtgaaatgat
 5641 aagatattcc tgtggcattt tagcttgaga gtctctgggt tctgggagat ttccctactc
 5701 ccttttaaaa tgcgaaggca cctataattt gcattgtggg tgtttacag ttgtctct
 5761 tagccctctt tcattggaga agaatcatg ggtatgccc agcgttttcc tgttctttg
 5821 cttccttgc acttgggtg tgggtgac ctggcaggat acagcaggag ttttctgc
 40 5881 attcatgtg gtccctgaa tagccactag gtatcttgg gaattgcaca atctagcgaa
 5941 accactgaag ttcatcatt cgtttact gacttcaga taatcggagt caaccttcta
 6001 acctctagt ctacttctt ccaataata ctgtacagac tggggagaat tattctacc
 6061 actccctcat ttcatgctt tctgccttc tctgaaggc tctatgatg aaaattgcaa
 6121 aaaccagct agtgcttct cctgttctt cattcaaat aggaagatgg tcatacctaa
 45 6181 tgtcttggg atcatggata gaatatgtg agaagacata gaaatagcct aattaaagg
 6241 cacagcatga ctgcaactt tacaatcat tgaataaag ttactttag gtgattgtg
 6301 ttttctatt ctttcttca tttaataat atccagctc tgccatttt gccacgggtc
 6361 aatgttgtat gcattgact ttaggttat aaatacatca gaatgttctt tgcgcctc

6421 cttacatgga aaactcaca accacctata caatccaaaa gaggggaaac agctcatctc
 6481 atattaatta tgggccattt ccatgatagg atattatgca accattaaaa atcatatttt
 6541 atctgagtgt tagtatatgg attatttta ttatttatac ttttcagtgt aattctctc
 6601 tgagcatact acttttgag tiaggaaac aaatcagtaa atgcattttt ttttttaac
 5 6661 aaaaactaaa gcacctgggt ttttgagta tagttccag gtgcgccaca ggccttctga
 6721 aacgtttcc ttcggatttt tcttcccca ggtgagctgt caggagagaa tgtcgaaca
 6781 ggtcaagaga tgtctgtc aggttccag acatgtgcc gggaggtgct tcagtatctg
 6841 gccaagcac agaactcgc ggacctgaag tcttcgcagc ttgcacca cctccaccgg
 6901 gtggtctcgg agctgctgca ggtggtacc tccaggaagc catcagacc agtcccaaa
 10 6961 gtgatggact tcaaggaaaa acccagctct cggccaaag gtccggaagg tcttgggaaa
 7021 aactgcgtgc cagtcatca gcggacttc gtcactcga gtggggagca gagcggcagc
 7081 gacacggca cagacagtgg ctatggagga gaatcgaga agggcgactt gcgcagtgag
 7141 cagccgtgct tcaaaagtga ccacggacgc aggttcacga tgggagaaag gatcggcgca
 7201 attagcaag agtccgaaga acccccaca aaaaagaacc ggatgcagct ttcggatgat
 15 7261 gaaggccatt tctactagc tgacctgac agtccccgt tctggggccc acaccacac
 7321 cagcctcct tctgcctgcc ctctacctg atccacctt cagcactgc ctacctgcc
 7381 atgctggaga agtgcctgga tcccactca gtccagtgc tataccagg cctcaacgcc
 7441 tctccgcag cctctctag ctcatgaac ccagacaaga tctcgctcc ctgtctatg
 7501 cccagagac tcccttctc ctgccagct catcgctcg tgcacttc tgtctgtc
 20 7561 caagctctga agccaatccc cctttaaac ttgaaacca aagactaac tcttagggg
 7621 atcctgctgc tttgcttcc ttcctcgta ctctaaaa agcaaaaa aagttttgt
 7681 gaatgctga agattgtgc attgtgata ctgagataat ctgaggcatg gagagcagat
 7741 tcagggtgtg tgtgtgtg tgtgtgtg tgtgtgtg cgtgtgcgtg cacatgtgtg
 7801 cctgcgtgtt ggtataggac tttaaagctc ctttggcat agggagtc cgaaggattg
 25 7861 ctgacatca ggagacttg gggggattgt agcagacgtc tgggctttc cccaccaga
 7921 gaatagcccc ctgcataca catcagctgg atttcaaaa gcttcaaagt ctgtctgt
 7981 gagtactct tcagttggg agctgggtct gtggcttga tcaagagga ctttcaaaag
 8041 agggctttc agggctcagc tccaaccag ctgtaggac cccaccctt tgccttatt
 8101 gtcgactga ctaccagac gtcggggaga gagagcagtc agaccagct tctgctaac
 30 8161 atggggagggt agcaggcact ggcatagcac ggtagtggt tggggagggt tccgcagtc
 8221 tgcctccac cctgcctcg gaagaataa gagaatgtat tccctactc aggccttctg
 8281 agtgattagc ttactaagga actgaaatg gcccctgt acaagctgag ctgcccggga
 8341 gggagggagg agttccctgg gcttctgga cctgttcta ggcctaacca ttagtacta
 8401 ctgtgcaggg aaccaaacca aggtctgaga aatgcggaca ccccgagcga gcacccaaa
 35 8461 gtgcacaaag ctgagtaaaa agctgcccc tcaaacaga actagactca gtttcaatt
 8521 ccactctaaa actccttta accaagctta gcttctcaa ggcctaacca agccttggca
 8581 ccgccagatc ctttctgtg gctaattct ctgccaac ggcataatga gtgtcctat
 8641 tgctaaaaag gattcgtct cttcaaga agtttatt ttggtccaga gtactgttt
 8701 tcccagtggt tccagccagc tccgcagcag ctttcaara tgcactatgc ctgattgctg
 40 8761 atcgtgttt aacttttct tttctgtt ttatttgggt attagtcgt tgccttatt
 8821 tgtaaagctg ttataatat atattatata aatatatta aaaggaaaat gttcagatg
 8881 ttatttga taattactg attcacacag tgagaaaaa tgaatgtatt cctgttttg
 8941 aagagaagaa taattttt ttctctagg gagaggtaca gtgttatat ttggagcct
 9001 tctgaagggt gtaaaatgt aaatatttt atctatgagt aaatgttaag tagttgttt
 45 9061 aaaactacta ataaaataat tctttcctg tgaagagaa agatggtctc ctgagtttt
 9121 taaaatagct cacttggtgt gataggctg actccatgt cctcttct tggctgaagc
 9181 aaaccagta atctcagtgt aattcattc agcttcat gtgcttga itcaatcaga
 9241 ataagctag tcaacctggc aggtaaatat tggttcaatt ttgttaac ccttgcagg

1. **Introduction**
 2. **Background**
 3. **Methodology**
 4. **Results**
 5. **Conclusion**
 6. **References**

-58-

9301 gggtcatgat catcctctac cttcttcag agttaagtaa aaagcatatt ttcccttgcc

9361 ctgctttcat cg

1
 2
 3
 4
 5
 6
 7
 8
 9
 10
 11
 12
 13
 14
 15
 16
 17
 18
 19
 20
 21
 22
 23
 24
 25
 26
 27
 28
 29
 30
 31
 32
 33
 34
 35
 36
 37
 38
 39
 40
 41
 42
 43
 44
 45
 46
 47
 48
 49
 50
 51
 52
 53
 54
 55
 56
 57
 58
 59
 60
 61
 62
 63
 64
 65
 66
 67
 68
 69
 70
 71
 72
 73
 74
 75
 76
 77
 78
 79
 80
 81
 82
 83
 84
 85
 86
 87
 88
 89
 90
 91
 92
 93
 94
 95
 96
 97
 98
 99
 100
 101
 102
 103
 104
 105
 106
 107
 108
 109
 110
 111
 112
 113
 114
 115
 116
 117
 118
 119
 120
 121
 122
 123
 124
 125
 126
 127
 128
 129
 130
 131
 132
 133
 134
 135
 136
 137
 138
 139
 140
 141
 142
 143
 144
 145
 146
 147
 148
 149
 150
 151
 152
 153
 154
 155
 156
 157
 158
 159
 160
 161
 162
 163
 164
 165
 166
 167
 168
 169
 170
 171
 172
 173
 174
 175
 176
 177
 178
 179
 180
 181
 182
 183
 184
 185
 186
 187
 188
 189
 190
 191
 192
 193
 194
 195
 196
 197
 198
 199
 200
 201
 202
 203
 204
 205
 206
 207
 208
 209
 210
 211
 212
 213
 214
 215
 216
 217
 218
 219
 220
 221
 222
 223
 224
 225
 226
 227
 228
 229
 230
 231
 232
 233
 234
 235
 236
 237
 238
 239
 240
 241
 242
 243
 244
 245
 246
 247
 248
 249
 250
 251
 252
 253
 254
 255
 256
 257
 258
 259
 260
 261
 262
 263
 264
 265
 266
 267
 268
 269
 270
 271
 272
 273
 274
 275
 276
 277
 278
 279
 280
 281
 282
 283
 284
 285
 286
 287
 288
 289
 290
 291
 292
 293
 294
 295
 296
 297
 298
 299
 300
 301
 302
 303
 304
 305
 306
 307
 308
 309
 310
 311
 312
 313
 314
 315
 316
 317
 318
 319
 320
 321
 322
 323
 324
 325
 326
 327
 328
 329
 330
 331
 332
 333
 334
 335
 336
 337
 338
 339
 340
 341
 342
 343
 344
 345
 346
 347
 348
 349
 350
 351
 352
 353
 354
 355
 356
 357
 358
 359
 360
 361
 362
 363
 364
 365
 366
 367
 368
 369
 370
 371
 372
 373
 374
 375
 376
 377
 378
 379
 380
 381
 382
 383
 384
 385
 386
 387
 388
 389
 390
 391
 392
 393
 394
 395
 396
 397
 398
 399
 400
 401
 402
 403
 404
 405
 406
 407
 408
 409
 410
 411
 412
 413
 414
 415
 416
 417
 418
 419
 420
 421
 422
 423
 424
 425
 426
 427
 428
 429
 430
 431
 432
 433
 434
 435
 436
 437
 438
 439
 440
 441
 442
 443
 444
 445
 446
 447
 448
 449
 450
 451
 452
 453
 454
 455
 456
 457
 458
 459
 460
 461
 462
 463
 464
 465
 466
 467
 468
 469
 470
 471
 472
 473
 474
 475
 476
 477
 478
 479
 480
 481
 482
 483
 484
 485
 486
 487
 488
 489
 490
 491
 492
 493
 494
 495
 496
 497
 498
 499
 500
 501
 502
 503
 504
 505
 506
 507
 508
 509
 510
 511
 512
 513
 514
 515
 516
 517
 518
 519
 520
 521
 522
 523
 524
 525

Figure 1

Figure 1

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☒ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.